ABSTRACT

BIOLOGY

WARD, DORIS J. B.S. RUST COLLEGE, 1985

INVESTIGATION OF THE MECHANISMS OF ESTRADIOL INSENSITIVITY IN THE FEMALE SQUIRREL MONKEY

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Dissertation dated May, 1993

The reproductively active female squirrel monkey is characterized by markedly elevated plasma estradiol levels. Plasma estradiol levels have been reported to be 10 to 30 times that in Old World primates and humans. However, the exact mechanisms responsible for these elevated estradiol concentrations have not been fully elucidated. In this study, two possible mechanisms contributing to elevated steroid levels were investigated. Investigation of uterine estrogen receptor binding parameters using the nuclear exchange method, revealed a uterine receptor concentration of 228 ±17.6 pmol/l and a dissociation constant of 2.80 ±0.24 nM. Nuclear receptor levels were found to be 46.0 ±19.4 pmol/l with a dissociation constant of 5.42 ±1.92 nM.

Intravenous administration of [^{3}H]-estradiol, followed by subsequent collection of urine and feces at 24 h intervals for a duration of 96 h, revealed that 71.4 % of the administered radiolabeled estradiol was recovered in the feces with only a 4.23 % recovery of radiolabeled estrogen in the urinary pool. Subsequent isolation and identification of estrogen metabolites by standard procedures including ion exchange chromatography and TLC, revealed that sulfate conjugates represented the major metabolites, with $E_2$17S accounting for over 55% of the total administered dose. Recovery of glucosiduronate conjugates in both urine and feces was minimal. Subsequent in vitro liver preparation, that involved characterization of E_2-17ß sulfo-transferase activity in cytosol fractions, revealed that the female squirrel monkey may possess a sulfotransferase that is specific for estradiol sulfation at the C-17ß position. This enzyme could in effect reduce estrogenicity by masking the C-17 hydroxyl group necessary for activity.

INVESTIGATION OF THE MECHANISMS OF ESTRADIOL INSENSITIVITY IN THE FEMALE SQUIRREL MONKEY

A DISSERTATION

SUBMITTED TO THE FACULTY OF CLARK ATLANTA UNIVERSITY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

BY

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ATLANTA, GEORGIA

MAY 1993

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ACKNOWLEDGMENTS

This dissertation is dedicated with love and gratitude to the memory of Sammie McCaskill, Sr., the extraordinary man who first inspired me, to my mother Ella M. Ward, and other family members whose strength and love sustained me in this as in so much else.

I would like to extend special thanks to Dr. Paul I. Musey for his patience, guidance, and understanding. To Dr. Juarine Stewart, Dr. John Browne, and Dr. William Dashek, members of my dissertation committee, my sincerest appreciation is extended. I would also like to thank Mrs. Joyce Lockhart and other staff members of the Biology Department of Clark Atlanta University.

Finally, special thanks to sincere friends for their support and enthusiasm.

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LIST OF ABBREVIATIONS

BW----------	Body Weight
ß/ase----------	ß-glucuronidase
ACTH----------	Adrenocorticotrophic hormone
CBG----------	Cortisol binding globulin
DEAE----------	Diethylaminoethyl
DHA----------	5α-Androstane-3ß,17ß-diol
DHEA----------	Dehydroepiandrosterone
DTT----------	Dithiothreitol
DBP----------	Vitamin D_3 Binding Protein
E_1----------	Estrone (1,3.5,[10]-Estratriene-3-ol-17-one
E_2----------	Estradiol-17ß (1,3,5,[10]-Estratriene-3,17ß-diol)
E_3----------	Estriol (1,3,5-[10]-Estratriene-3,16α,17ß-triol)
$E_2$17S----------	estradiol-17S-sulfate
E_1G----------	estrone glucosiduronate
E_2G----------	estradiol glucosiduronate
$E_2$3,17diS----------	estradiol-3,17ß disulfate
ER----------	Estrogen Receptor
HAP----------	Hydroxylapatite

FSH----------	Follicle Stimulating Hormone
GLC----------	Glycolithocholate
LH----------	Lutenizing Hormone
MCR----------	Metabolic Clearance Rate
PAPS----------	Adenosine-3'-phosphate-5'-phosphosulfate
PAP----------	Adenosine-3',5'-diphosphate
s/ase----------	phenolsulfatase
SH----------	sulfhydryl group
SHBG----------	Sex Hormone Binding Globulin
TEDG----------	Tris-HCl buffer with ethylenediamine tetraacetic acid, dithiothreitol,and 10% glycerol,pH 7.4
UDPGA----------	Uridine-5'-Diphosphoglucoronic Acid

CHAPTER I

INTRODUCTION

Traditionally, species of the Old World primates, including the rhesus and the cynomolgus monkeys, have been used as models for human reproductive and physiologic studies. Similarities in physiology of the reproductive tract as well as similarity of hormonal regulation of menstrual cycle and pregnancy warranted their use for such studies. However, due to limited availability and increasing cost of maintenance, alternative models have been sought.

The squirrel monkey (Saimiri sciureus), a representative New World primate, has been investigated as an alternative model for human reproductive physiology studies. The New World primates are more adaptable to laboratory procedures because of their small size. Besides, the cost for use of these primates is much lower than for other primates. In addition, several aspects of their reproductive physiology were applicable to humans. However, initial investigations showed that in some aspects, like the reproductive physiology, these primates differed significantly from Old World primates and humans.

The New World primates examined thus far exhibit an extreme seasonality of breeding (Dukelow, 1981), accompanied by fluctuating level of gonadal hormones (Wolf et al., 1977). Another difference of note is the fact that the New World primates do not demonstrate a conspicuous estrus cycle characteristic of lower primates, nor a detectable menstrual cycle characteristic of higher primates. Additionally, based upon vaginal cytology (Hutchinson, 1977) and behavioral observations (Abbot and Hearn, 1978), and measurement of steroid hormone plasma levels (Wolf et al., 1977), investigators have demonstrated that the mean ovarian cycle length is eight to ten days. This is in sharp contrast to the twenty-eight day menstrual cycle (Stevens et al., 1970; Knobil, 1974) characteristic of the Old World primates and humans. Another difference revealed is the markedly elevated levels of steroid hormones. All the New World primates examined thus far are characterized by high circulating levels of adrenal and gonadal hormones while maintaining comparatively normal levels of peptide hormones (Hearn, 1983). In the New World primates, plasma levels of glucocorticoids (Coe et al., 985; Setchell et al., 1977) and mineralcoids (Brown et al., 1970) were found to be elevated as well. Other preliminary investigations indicated initially that the pathways of steroid excretion and metabolism may be different in the New World primates (Setchell, 1977).

The reproductively active female squirrel monkey, a representative species of the New World primates, has characteristically elevated circulating levels of estrogens. The plasma estrogen level has been reported to be at least 10 to 30 times that in Old World primates and humans (Dukelow, 1981). Some investigators have even reported plasma estrogen levels as high as 100 times that of Old World primates (Wolf et al., 1977). This markedly elevated estrogen profile presented an enigma to the biological community, for the estrogen profile did not appropriately reflect the physiologic state. Despite the elevated plasma estrogen levels, these primates did not exhibit any apparent abnormalities in their reproductive physiology (Diamond et al., 1984). In addition, examination of target tissues and organs revealed no adverse changes in the morphology of these structures. Furthermore, normality of reproductive organs was evidenced in the fact that mature females were capable of bearing offspring.

One explanation could be that the estrogen receptor (ER) binding parameters are altered, leading to end-organ resistance. Chrousos et al. (1984) have reported a lower uterine cytosol unoccupied ER level in squirrel monkeys compared to cynomolgus monkeys. Medlock et al. (1981) have shown that pharmacological levels of estradiol down

regulate the rat uterine ER. In the squirrel monkey, low receptor levels could provide normal responses in the presence of high estradiol levels. Since estrogen binding to its receptor is dependent upon both hormone and receptor concentration, lowered levels of either will reduce responses. Therefore, squirrel monkey ER could have a lower dissociation constant (K_d). A higher estrogen concentration would then be needed to saturate the receptor and lead to tissue responses.

In order to draw an accurate picture of this relation between steroid binding and physiological responses, different techniques have been used to determine the total receptor concentration and K_d in the cytosol and nuclear compartments. The exchange assays (Anderson et al., 1972a) carried out at 30-37°C, have allowed measurement of ER occupied by E_2 as well as unoccupied ER.

Apparent resistance to other steroid hormones including cortisol (Chrousos et al., 1982a), progesterone (Keller et al., 1979; Chrousos et al., 1982b) and 1,25-dihydroxyvitamin D (Eil et al., 1981) has been described recently in humans and New World primates. In all of these syndromes characterized by normal physiological function, elevated plasma steroids concentrations have been attributed to abnormalities in steroid receptor affinity or concentration. The response of a cell to a hormone depends, however, on a number of factors in addition to

receptor affinity and concentration. The currently accepted model of steroid action states that the magnitude of the signal to the cell is dependent upon the concentration of receptor which in turn is dependent upon the concentration of receptor, its affinity, and the concentration of bioactive hormone (Clarke, 1926). As the concentration of bioactive estrogens is dependent upon its rate of metabolism, an alternative explanation for high plasma estrogen levels may be due to a low clearance rate. Because of the unusual features of estrogen physiology in this species, a detailed study of the pattern of urinary and fecal estrogen excretion following $[^3H]$-E_2 administration was undertaken.

The elevated plasma estrogen concentrations in the squirrel monkey are possibly caused by factors that exert their influence prior to interaction with the intracellular receptor. The squirrel monkey and other New World primates might exhibit markedly elevated plasma estrogen levels due to binding with plasma proteins. When steroids are released into systemic circulation, they circulate primarily bound to specific plasma proteins or nonspecifically to albumin. According to the consensus, only that fraction that is free and unbound to plasma proteins is available for the elicitation of characteristic hormonal response (Clarke, 1926). In plasma, only a minute fraction of hormone is free and readily diffuses out of the

capillaries for interaction with target receptors. Therefore the rate of dissociation or the concentration of steroid binders in plasma can influence the level of bioactive steroids.

Previous reports by Musey and coworkers (1982) have shown that in the squirrel monkey, sulfoconjugates are the major plasma metabolites. Following intravenous administration of [^{3}H]-E_2, more than 98% of the radioactivity in plasma was identified as $E_2$17S and $E_2$3,17S. Furthermore, results in the present study show that these two metabolites are the predominant estrogens excreted in urine and feces. Combined, these findings suggest that this species might possess an enzyme system that is more efficient in the sulfation of estradiol. It is possible that in the squirrel monkey 17ß sulfation of E_2 is an adaptive mechanism for reducing estrogenicity. Pack and Brooks (1974) have postulated that sulfoconjugation may play a role in the regulation of responsiveness to target tissues by governing the concentration of free E_2. The presence of relatively high concentrations of estradiol-17-sulfate in body fluids of the squirrel monkey and its implications in regulation of E_2 responsiveness warranted the characterization and partial purification of the conjugation enzyme responsible for the formation of these metabolites.

CHAPTER II

REVIEW OF LITERATURE

Estrogens: A General Overview

Research on estrogens began around 1900 when Halban (1900) and Knauer (1900) independently implanted rabbit ovaries into previously ovariectomized rabbits and concluded that these organs stimulated uterine growth by secretion of a biologically active substance into the blood. The estrogens are naturally occurring steroid hormones that coordinate systemic responses of the female reproductive physiology. Estrogens are responsible for the development of female secondary sex characteristics. In addition, estrogens, in conjunction with other gonadal hormones, play a pivotal role in the regulation of cyclic patterns of the female including ovum maturation, ovulation, and the menstrual flow.

Estrogens belong to a subclass of lipids that contain a basic skeletal structure of four fused rings referred to as perhydrocyclo-pentanophenanthrene. All naturally occurring estrogens are C_{18} steroids with a characteristic angular methyl group at position 18, an aromatic A ring, and a phenolic group at position 3. Because they were the first estrogens to be isolated, estrone, estradiol-17ß, and

estriol are regarded as the three classical estrogens. Estradiol-17ß is the hormone normally secreted by ovarian follicles and since it has the greatest affinity for the estrogen receptor, it is regarded as the principally active natural estrogen.

Metabolism of Estrogens

The first indication of estradiol metabolism was evidenced by the demonstration of quantitative amounts of estrone in urine following administration of estradiol (Heard and Hoffman, 1941). Fishman et al. (1960) further investigated the in vivo metabolism of [^{3}H]-estradiol and [^{14}C]-estrone in the human. The [^{14}C]/[^{3}H](3:1) ratios for the estrogens and their metabolites from blood and urine led the authors to the conclusion that the oxidative conversion of estradiol-17ß to estrone exceeded the reverse reaction, leading to the conversion of estrone to estradiol 17ß. This group also postulated that while estradiol was the primary estrogen secreted by the ovaries, conversion to estrone represented the major circulating form of the estrogen. Diczfalusy (1961) later drew similar conclusions utilizing various liver tissue preparations.

With the appearance of more sophisticated analytical tools, detection of other quantitatively important estrogens was made possible. Marrian (1959) established the concept of metabolism of estradiol at ring D with the

identification of a 16-oxygenated estrogen in urine and serum. Fishman et al. (1960) demonstrated the 2-hydroxylated metabolites of estrogens in urine and plasma following the intravenous administration of tritiated estradiol and estrone. In addition, hydroxylations were shown to occur at the aromatic C-4 position (Gelbke et al., 1977) and the cyclo-aliphatic positions, 6, 7, 11, 14, 15, and 18 (Diczfalusy and Lauritzen, 1961; Breuer and Nocke, 1960). Hydroxylations at positions 2 and 16 represent major pathways of estrogen metabolism in man (Fishman et al., 1959).

Conjugation Reactions

Inactivation of estrogens occurs primarily in the liver, and the inactive metabolites are generally conjugated as sulfate esters or ß-glucosiduronates (Briggs and Brotherton, 1970) before excretion in urine. At least two systems have been described that are active in the formation of sulfate esters of estradiol. Adams et al. (1967, 1974) described a sulfotransferase that was specific for estrogen while Ryan and Carroll (1976), and Gugler et al.,(1970) independently characterized a sulfotransferase that is rather nonspecific for 3 ß-hydroxysteroids. The steroid sulfates are formed by the action of a soluble sulfokinase that uses 3'-phosphoadenosine-5'-phosphosulfate (PAPS) as the sulfate donor. Glucosiduronates are formed

by the action of a glucuronyl transferase present primarily in liver microsomes, and uridine diphosphoglucuronic acid (UDPGA) that serves as the glucosiduronate donor (Barman, 1969). Steroid hormones have also been shown to undergo conjugation reaction with glutathione (Jellinck et al., 1965), N-acetylglucosamine (Collins et al., 1968), and glucose (Layne, 1971).

In the past, it was believed that the purpose of the conjugation was to render bioreactive compounds physiologically less active and more water soluble to facilitate rapid excretion. However, recent studies have led to the idea that conjugates, particularly sulfates, may play a pivotal role in steroid activity. Under normal conditions, the metabolic clearance rate of estrone sulfates is less than 10% of that of unconjugated estrone (Rosenthal et al., 1972). In addition, a number of target estrogen tissues, including the uterus (Zuckerman and Hagerman, 1969; Dolly et al., 1972) and the pituitary (Payne et al., 1973) contain sulfatases capable of hydrolyzing the sulfate moiety, thus rendering the estrogen biologically active. Estrone sulfate may serve as a transport form of estrogen until reaching its target site (Ruder et al., 1972). Baulieu and Dray (1963) demonstrated the direct conversion of estrone sulfate to estradiol sulfate without prior hydrolysis of the sulfate moiety.

Upon delivery to the target site, the estrogen sulfate may be reactivated immediately by sulfatases present in target tissues.

Patterns of Excretion

The fate of intravenously administered estrogens and metabolites of estrogen have been extensively studied in humans and to a lesser extent in subhuman primates. Very small proportions of steroids are excreted unchanged in the urine and bile. The bulk undergoes chemical modifications, mainly oxidative or reductive reactions, followed by conjugation with either sulfuric or glucosiduronic acids.

In the human, estrogen metabolites are principally eliminated by way of the kidney. In vivo studies by Sandberg and Slaunwhite (1957) demonstrated that following administration of tritiated estrone and estradiol, more than 80% of radiolabeled estrogens were eventually recovered in the urine. Migeon et al. (1959) noted similar results when [^{14}C]-estrone was administered to human subjects. Only 1-2% of the estrogens appeared as unconjugated steroids with 5% as sulfate esters and 33-53% as glucosiduronates.

Graham et al. (1972) have studied urinary excretion of estrogen metabolites in the chimpanzee. The free, unconjugated fraction accounted for approximately 20% of the total urinary estrogens. Sulfates and glucosiduronates accounted for 7 and 75%, respectively

(Graham et al., 1972). In the orangutan, 17% of the total urinary estrogens was identified as unconjugated estrogens. Again the glucosiduronates were the major metabolites (51%), with sulfates representing 34% of the total urinary estrogens. An interesting feature of urinary metabolites in apes was the increased excretion of free, unconjugated estrogens when compared to the urinary excretion of unconjugated estrogens in women (Crowell et al., 1967). However, in apes and women, estrone glucosiduronate was consistently the major urinary estrogen conjugate (Crowell et al., 1967; Wright et al., 1978).

Sandberg and Slaunwhite (1965) were the first to establish the importance of biliary excretion in estrogen inactivation. They showed that when radiolabeled estradiol was administered to patients in whom biliary T tube were inserted, more than 50% of the radiolabeled steroids were recovered in the bile. In these patients, only 15% of the radioactivity was accounted for in the urine. Adlercreutz et al. (1982) have suggested a concentrating mechanism that occurs when steroids are transferred from the hepatic cells to the bile canaliculi, since the concentration of individual steroids in the bile was shown to be very much higher than their precursor hormone in plasma. Thus, the extent to which a steroid is sequestered in bile would influence its rate of elimination from circulation.

Factors modulating sequestration of steroids in bile are not completely known, but molecular size and structure of metabolites seem to influence biliary secretion. Musey et al. (1972) showed that double conjugates of estrogens such as estriol-3-sulfate-16-glucosiduronate and estradiol-3-sulfate-17-glucosiduronate are preferentially excreted into the bile while monoglucosiduronates (Emerman et al., 1965, Jirku et al., 1971) such as estrone glucosiduronates and estriol-3-glucosiduronates are excreted primarily in the urine. In pregnant women, estriol and 16α-hydroxyestrone are the major estrogens in bile (Adlercreutz et al., 1964). Estrone metabolites excreted in bile are predominantly sulfate esters whereas estradiol is a relatively minor estrogen in bile (Adlercreutz and Martin, 1976).

Fecal excretion represents a minor route of elimination of estrogens. Only 1-18% of intravenously administered estrone and estradiol is excreted in feces (Sandberg and Slaunwhite, (1957). The estrogens recovered in feces are unconjugated estrogens, primarily estradiol, estrone and estriol (Eriksson and Gustafsson, 1971). In addition to these three major estrogens, Adlercreutz and Jarvenpaa (1982) tentatively identified 16α- and 16ß-hydroxyestrone, 3-methoxyestrone and estradiol-17α in fecal extracts of humans.

Enterohepatic Circulation

Extensive biliary excretion of estrogens results in a prolonged enterohepatic circulation that is responsible for its delayed clearance in urine and feces. The importance of this phenomenon was first suggested by Cantarow (1943) based upon the demonstration in dogs of a high estrogenic activity of mesenteric blood following instillation of estradiol into intestinal loops and the high estrogen excretion into the bile following duodenal administration of estradiol. The quantitative significance was later demonstrated by Sandberg and Slaunwhite (1965) when it was observed that, although large percentages of radiolabeled estrogens were excreted in bile, only 10% of the labeled steroids appeared in the feces. Subsequently, the work of many investigators showed that most steroids undergo conjugation by the liver and some return to the circulation before renal excretion. Other metabolites cross the membranes of hepatic canaliculi to be excreted in the bile followed by secretion into the small intestine. Enterohepatic circulation of estrogen in man has been extensively reviewed (Sandberg and Slaunwhite, 1957, 1965; Emerman et al., 1967; Jirku et al., 1974). In the intestine, steroid conjugates are hydrolyzed, reabsorbed by the gut mucosa, and then are transported back to the liver for another round of metabolism as described above.

Factors Affecting Estrogen Metabolism

The pattern of estrogen metabolism and excretion may be altered by various factors. Zumoff et al. (1968) demonstrated that men with cirrhosis of the liver excreted a greater amount of radiolabeled estrogens in urine than men with relatively normal livers. Urinary excretion of estriol was also increased in cirrhotic patients (Cameron, 1957). In addition, Hellman et al. (1956, 1970) showed that following intravenous injection of estradiol in patients with total biliary obstruction, 48% of the radioactivity was excreted in 24 h compared to 34% in normal subjects. Moreover, 29% of the radioactivity was present in the non-glucosiduronate fraction as compared to only 12% in the normal human. Zumoff et al. (1968) also reported a variation in the pattern of estrogen metabolism in men with hypothyroidic conditions and men with breast cancer. In such patients ring A metabolites of estrone were decreased while there was an increase in 16α-hydroxylation (Fishman, 1965).

Changes in intestinal microflora attributable to antibiotic administration have also been shown to cause alterations in estrogen metabolism. Alterations in intestinal microflora affect a number of steroid modifications attributed to bacteria. These changes include hydroxylase, dehydroxylase, reductase, and epimerase activities (Eriksson et al., 1971). These

alterations result in the excretion of steroids in the feces that normally would have been excreted in the bile. Engel (1957) demonstrated that antibiotics reduce the percentage of radioactive 17ß-estradiol excreted in the urine. Adlercreutz et al. (1976) have also shown that ampicillin administration to pregnant women reduces the urinary excretion of endogenous 16-hydroxylated estrogen but causes an increase in the fecal excretion of both conjugated and unconjugated estriol.

The metabolism of steroids could likewise be influenced by hereditary predisposition and by chemicals that affect their metabolism. Conjugation is also dependent upon the availability of PAPS and UDPGA that are in turn related to the nutritional states (Slaunwhite et al., 1963; Hoskins and Zanecheck, 1968).

Transport of Steroid Hormones

In addition to receptor binding properties, the response of a cell to a hormone depends as well on factors that influence steroid dynamics and bioavailability. These parameters fluctuate as a function of several variables.

One factor that plays a significant role in the concentration of circulating hormone is the presence of steroid binding proteins in plasma. Early models of steroid hormone transport proposed that only the non-protein bound, i.e., the free steroid hormones were able to

influence biological activity (Recant and Riggs, 1952). However, the affinity of plasma proteins for steroids may determine the efficacy of the steroid in a biological system by regulating tissue uptake and metabolism.

Steroid hormones circulate, primarily not in the free form, but bound nonspecifically to albumin, or specifically to plasma proteins. The specific binding proteins bind with a relatively high affinity for their respective steroids: sex-hormone binding globulin (SHBG) for testosterone and estradiol; cortisol binding globulin (CBG) for cortisol, progesterone, and aldosterone; and vitamin-D-binding protein (DBP) for vitamin D_3. In man, approximately 97% of circulating testosterone is in the bound form with only about 3% circulating as free testosterone. Of the bound fraction, over 65% is bound to SHBG with a dissociation constant of 1.31 nM. Thirty-three percent of circulating testosterone is bound to albumin (Baird et al., 1969). Since estradiol has a slightly decreased binding affinity (K_d=1.40 nM) for SHBG than testosterone, binding to albumin (40%) is increased relative to SHBG (58%)(Siiteri et al., 1982). This general pattern is mirrored in androgen and estrogen dynamics in the baboon (Longcope et al., 1988). The plasma binding capacities for glucocorticoids are similar. Ninety percent of cortisol is bound to CBG (K_d=2.20 nM), whereas CBG binds only about 20% progesterone with 80% bound to albumin (Dunn et al., 1981).

Several investigators have studied several aspects of serum transport and steroid dynamics in New World primates with the hope of clarifying the nature of the dramatic differences in hormone levels that characterize this group of animals (Siiteri et al., 1982; Chrousos et al., 1982b; Pugeat et al., 1984a). The mean SHBG binding capacity of the New World primates was found to be generally lower than that of representative Old World primates (Pugeat et al., 1984). SHBG of New World primates, however, exhibited a lower binding affinity than that of Old World primates. Chrousos et al. (1984) reported CBG binding dissociation of 5.0±2.8 nM in the squirrel monkey while those of the rhesus monkey and man were 1.3±0.8 and 1.2±0.6, respectively. Despite the difference in SHBG binding parameters in this species, the plasma distribution of testosterone was found to be similar to that in the Old World primates with only about 3% circulating as free steroid and 97% bound to plasma proteins (Pugeat et al., 1984a).

Recently, Chrousos's group (Chrousos et al., 1984a) investigated CBG binding properties in the squirrel monkey. They reported major differences in CBG binding parameters compared to humans and other Old World primates. Not only were the total and free serum concentration of cortisol greatly elevated, but there were also variations in CBG binding capacity and considerable differences in CBG binding affinity for cortisol. The affinity constants for

squirrel monkey CBG were roughly 20-60 times lower than those in Old World primates. CBG binding capacity in the squirrel monkey was in the range of 150 to 300 nM compared to 15-40 nM in man. Consequently, the plasma free cortisol levels are 100 to 1000 fold greater in the squirrel monkey than plasma cortisol levels in humans (Brown et al., 1970).

No exact function has been assigned to plasma binding proteins as it relates directly to delivery of steroids to target sites. However, a strong correlation has been established between metabolic clearance rate (MCR) and the binding parameters of plasma binding proteins for their respective steroids. The average MCR for various steroids varies from 200 to 2000 liters per day, with variation dictated primarily by the concentration and affinity to which it is bound to a specific plasma-binding protein. The specific binding of testosterone and dihydroxytestosterone to SHBG and cortisol to CBG is reflected in relatively low MCRs for these steroids. Petra et al. (1985) have shown that when plasma SHBG was increased by infusion of SHBG into monkeys, thereby raising its plasma concentration considerably, the MCR of testosterone decreased. Unconjugated steroids which tend to be bound primarily to albumin, have relatively high MCRs. In humans, cortisol, which is predominantly bound to CBG, has a MCR of 200 liters per day and testosterone which is bound to a lower degree to SHBG, has a MCR of 500 liters

per day. Androstenedione and progesterone, which are predominantly bound by albumin, have a MCR of approximately 2000 liters per day (Wang et al., 1967; Vermeulen, 1986). In contrast, sulfate conjugates have a greater affinity for albumin and as a result reflect a lower MCR of about 20 liters per day (Sandberg et al., 1964). Of the conjugate steroids, sulfate conjugates are cleared from circulation more slowly than the glucosiduronate conjugates (VandeWiele et al., 1963).

Mechanism of Steroid Action

The characteristic effects of estrogen stimulus in target tissues have been described in detail (Mueller et al., 1958; Clark et al., 1976). Following administration of estrogen, a cascade of biological events ensue in target tissues including hyperplasia and hypertrophies, imbibition of water, and eventually increased cell division (Mueller et al., 1958). At the subcellular level, early events include phospholipid synthesis (Aizaway and Mueller, 1961), the production of an induced protein (Barnea and Gorski, 1970), an increase in the DNA-dependent RNA polymerase activity (Gorski, 1964), and increased nuclear RNA synthesis (Hamilton et al., 1968). After approximately six hours, ribonucleic acid accumulation occurs followed by protein synthesis, and after 24 h, an increased DNA synthesis is observed. The precise reason for the

tissue selectivity of estrogen action was, however, unknown before the discovery of a specific estrogen receptor.

In the early 1960s, Jensen and coworkers (Jensen and Jacobson, 1962) made a great contribution to the fundamental mechanisms of estrogen action. They were able to show the differential accumulation and retention of radiolabeled estradiol by the uterus, vagina, pituitary and other target organs (Jensen and Jacobson, 1962; Glasscock and Hoekstra, 1959). Subsequent studies by other investigators established the protein nature (Toft and Gorski, 1965) and intracellular localization of estrogen receptors (Jensen et al., 1968), the first hormonal receptors to be isolated and characterized. The estrogen-binding macromolecule was found to be an intracellular component of a proteinaceous nature that associated with the hormone by a strong non-covalent, physic-chemical attraction (Toft and Gorski, 1966). The approximate association constant for estradiol with its respective receptor is in the range of 10^{9}-10^{12} M^{-1} at 37°C (Erdos et al., 1969; Jensen et al., 1968). In its native state, i.e., unbound to hormone or isolation in the presence of low KCl, the sedimentation coefficient for the receptor was reported as 8S with a molecular weight of 236,000 daltons (Erdos, 1968; Korenman, 1968; Korenman and Rao, 1968). The 8S estrogen-binding complex can be dissociated into a 4.5 S

macromolecule by treatment with 0.3 M or higher concentration of KCl (Notides and Nielsen, 1974; Korenman and Rao, 1968). Upon binding to its respective hormone, followed by a temperature dependent translocation to the nucleus (Notides et al., 1975), there is a conformational change that results in a protein complex with a sedimentation coefficient of 5S and a molecular weight of approximately 110,000 daltons (Notides and Nielsen, 1974; Jensen et al., 1968).

Our current thinking of the biochemical events leading to a characteristic estrogen action has been influenced largely by the work of Jensen (1968) and Gorski (1968). In 1968, they independently proposed the now classical "two-step" model of steroid hormone intracellular interaction. According to this model, steroids diffuse into target cells and bind to cytoplasmic receptors in a strong but reversible association. Binding of estrogen to its specific receptor results in a conformational change of the receptor followed by a temperature dependent translocation of the "activated" complex to the nucleus. This activated receptor is now capable of high affinity binding to nucleus acceptor sites leading to modulation of transcriptional events.

Recent evidence has accumulated which raises questions about some aspects of the two-step model. The results of classical biochemical studies (Clark, 1984) as well as

recent histochemical reports utilizing autoradiographic (Stumpf et al., 1966), cytochemical (Nenci et al., 1981) and immunocytochemical (Govindan, 1980) techniques indicate that estrogen receptors are exclusively localized in the nucleus. It has been postulated that the isolation of cytoplasmic receptors is an *in vitro* artifact caused by cellular perturbation during homogenization (Walters et al., 1981). Notwithstanding these recent findings, the basic tenets of the classical model of hormone action remain valid, i.e., gene function is regulated as a direct consequence of the presence of active steroid hormone receptor complexes within target cell nuclei.

Receptor-Mediated Resistance

A compensatory increase in steroid hormone production more often than not accompanies syndromes of end organ resistance where there is partially or essentially complete loss of sensitivity to the effects of circulating steroids. In his initial description of complete testicular feminization syndrome, Morris (1953) noted that individuals with an apparently normal XY karyotype displayed a female external appearance but with ambiguous internal sex organs. Subsequently, Wilkins, (1957) demonstrated that these individuals had a profound resistance to the action of both

endogenous and exogenous androgens. In addition, plasma testosterone levels and rates of testosterone production by the testes were normal or in most cases higher than in normal men (Wilson and MacDonald, 1978).

Chrousos et al. (1982b) have also described a similar syndrome for glucocorticoid resistance in man. This syndrome is characterized by markedly elevated levels of plasma cortisol, increased production rates of cortisol, and elevated adrenocorticotrophic hormone (ACTH) levels accompanied by hypertensiveness and hypokalemia. However, there are no evidences of classical symptoms of Cushing's syndrome. Keller et al. (1979) have described progesterone resistance in humans. In this syndrome, resistance is characterized by abnormal endometrial biopsies during cycles in which the serum patterns of progesterone, estradiol, follicle stimulating hormone (FSH), and luteinizing hormone (LH) were shown to be normal. Administration of exogenous progesterone did not correct the abnormality. In <u>in vitro</u> studies of these tissues, the number of receptors in endometrial cytosol was half that of preparations from normal control subjects. Also a vitamin-D dependent rickets type II is associated with reduced nuclear uptake of the vitamin-D receptor complex in skin fibroblasts cultured from affected individuals (Eil et al., 1981). Similar conditions of partial resistance have been

described for aldosterone (Cheek and Perry, 1958) and for thyroid hormone (Refetoff et al., 1962, 1967). In the New World primates, end organ insensitivity to estrogen, progesterone, and other steroid hormones have been described, although these conditions are not accompanied by clinical manifestations of defects (Hearn, 1983; Chrousos et al., 1982a; Chrousos et al., 1984). In studies comparing estrogen receptor binding parameters in the squirrel monkey and the cynomolgus monkey, a representative species of the Old World primates, Chrousos et al.(1984a) reported uterine cytosol receptor concentrations in the squirrel monkey that were a third of that in the cynomolgus monkey. The progesterone receptor level in the squirrel monkey was about one-eighth the receptor level detected in the Old World monkey. However, the affinity constants were approximately the same in both Old and New World primates. Glucocorticoid insensitivity has also been described in the squirrel monkey as well as other New World primates. Chrousos et al. (1982a), using cultured skin fibroblasts, showed that similar concentrations of glucocorticoid receptors in both Old and New World primates; but a decreased binding affinity for dexamethasone in the New World species was reported compared to that in Old World primates.

Importance of Steroid Sulfation:

Steroid Sulfates in Steroid Biosynthesis

In the past, sulfation was thought only to be a prerequisite for rapid excretion. However, increasing evidence has warranted re-evaluation of this premise. A role for steroid sulfates in hormone synthesis was first suggested by the work of Lieberman's group (Calvin et al., 1963; Calvin and Lieberman, 1964; Roberts et al., 1961). These studies pointed out the existence of a pathway for the direct conversion of cholesterol sulfate to other sulfated steroids such as pregnenolone sulfate and dehydroepiandrosterone sulfate. Later Miyazaki et al. (1969) demonstrated that estrone sulfate was required for the conversion of estrone to catechol estrogens. Hobkirk and coworkers (1978) more recently have shown the importance of estrone sulfate in 16-hydroxylation of estrogen in mammalian liver. Payne and Singer (1979) have concluded that steroid sulfates serve as precursor pools for gonadal hormone synthesis in man.

Steroid Sulfates in Neoplasia

Abnormal accumulation of steroid sulfates has also been linked to cancer. The formation of steroid sulfates by adrenal tumors reported by Baulieu (1962) and Dao and Libby (1969) demonstrated that mammary tumor extracts exhibited elevated steroid sulfotransferase activity

compared to normal mammary tissue. Moreover, it was suggested that the sulfotransferase levels in the tumors could be correlated with the patient's response to adrenal ablation (Dao and Libby, 1969). In another in depth study of steroid transformation by neoplastic human breast, it was found (Dao et al., 1972) that malignant breast tumor tissue contained the enzymes necessary for transforming the precursor steroid dehydroepiandrosterone sulfate into estrogens. Furthermore, Dao et al. (1974) have shown that mammary tumor extracts contain sulfatases capable of hydrolysis of the sulfate moieties. Since exposure to estrogen has been implicated in the development of adenocarcinoma (Henderson et al., 1977), it is reasonable to suspect that estradiol and estrone sulfates play a role in the etiology of various cancers of female reproductive organs.

Properties of Various Sulfotransferases

Gregory and Lipmann (1957) were the first to provide proof for the existence of specificity of steroid sulfotransferase activity by the partial resolution of phenolic sulfotransferase activity from that of sulfotransferases that catalyze the transfer of a sulfate group donated by 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to dehydroepiandrosterone (DHEA) as well as to other 3ß-hydroxysteroids. Later, Nose and Lipmann (1958)

distinguished steroid sulfotransferase activity toward estrogens and DHEA. Shortly afterwards, Banarjee and Roy (1966) purified estrogen and DHEA sulfotransferases from guinea pig liver 70-fold and 20-fold, respectively.

Current evidence indicates that there may be at least six different steroid sulfotransferases. In 1974, Adams' laboratory reported the purification of an estrogen sulfotransferase from bovine adrenal (Adams and Poulos, 1967), ovary (Adams and Chulavatnatol, 1967), and the placenta (Adams et al., 1974). The enzyme was specific for estrogens and sulfated only the phenolic hydroxyl group of these compounds. The pH optimum was found to be between 8.0 and 10.0. The K_m of the estrogen sulfotransferase for PAPS was 37 µM, and that for estrone was reported to be 15 µM. A second steroid sulfotransferase described is the 3ß-hydroxysteroid sulfotransferase of rat liver (Ryan and Carroll 1976), human liver (Gugler et al, 1970), and human adrenal (Adams & MacDonald, 1979). Using DHEA as substrate, the 60-fold purified 3ß-hydroxysteroid sulfotransferase exhibited a pH optimum of 5.0, and the K_ms for PAPS and DHEA were 13 µM and 6 µM, respectively. It was specific for the 3ß-hydroxysteroids and, unlike the estrogen sulfotransferase, it exhibited simple Michaelis-Menten kinetics.

Chen et al. (1977) reported another steroid sulfotransferase that was specific for bile acids. Upon

35-fold purification from rat liver cytosol, its apparent K_m values for PAPS and taurolithcholate were 8 µM and 50 µM, respectively. The pH optimum was reported to be 6.5 with several essential sulfhydryl(SH) groups present in this 130,000 dalton protein. Recently, Chen et al.(1985) have purified bile salt sulfotransferase from human liver. Using glycolithocholate (GLC) as sulfated acceptor, the pH optimum was 6.5. The K_ms for PAPS and GLC were 0.7 µM and 2.0 µM, respectively. This sulfotransferase also required the presence of a sulfhydryl group.

Singer et al. (1976) reported on three sulfotransferases isolated from rat liver that sulfated adrenal glucocorticoids. The enzymes were designated sulfotransferases I, II, and III based on the order of their elution from a DEAE-Sephadex A-50 column. Sulfotransferase III appeared to be the 3-ß hydroxysteroid sulfotransferase characterized by Ryan and Carroll (1976). Sulfotransferase II was purified approximately 2500-fold from liver homogenates of female rats. The K_ms for PAPS and cortisol were 6.82 µM and 6.28 µM, respectively. A pH optimum of 6.0 was obtained. Sulfotransferase I was purified 1250-fold and exhibited a pH optimum of 6.0 using cortisol as a substrate. The K_ms for cortisol and PAPS were 7.09 µM and 10.6 µM, respectively. Although these two enzymes, sulfotransferase I and II, exhibited similar

properties, they differed greatly in substrate preference, Km for PAPS, and the response to metal ions. Recently, Lewis et al. (1981) have also reported the isolation of a new mineralcorticoid sulfotransferase from the liver of female rats.

The isolation and characterization of at least six steroid sulfotransferases that have specific steroid substrate requirements seem to strongly support the claim that sulfation is more than a general mechanism of steroid detoxification. Steroid sulfation may play a role in the regulation of hormone action in a more specific manner than originally postulated.

CHAPTER III

MATERIALS AND METHODS

Materials

17ß-[2,4,6,7^{3}H]-Estradiol (E_2) (specific activity 104 Ci/mmol) with greater than 98% radiochemical purity was obtained from Radiochemical Center, (Amersham, U.K.). Diethylstilbestrol was purchased from Research Plus Steroid Laboratories Inc. (Denville, NJ). Unlabeled 17ß-estradiol, 17α-estradiol, estrone, estriol, DHEA, testosterone, E_23S, and PAPS were all purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of analytical grade.

Animals

Six young non-cycling female squirrel monkeys (Saimiri sciureus) were obtained from Primate Services, Inc. Miami, FL. They were housed in temperature (23°C) and humidity (50%) controlled rooms with a 12 h light: 12 h dark cycle. Purina Monkey Chow (Ralston Purina Company) and water were provided ad libitum. Two weeks after arrival, estradiol was administered (i.m. 10 ug/kg BW once daily for two days) to three monkeys and vehicle (sesame oil) to the remaining three monkeys. Uteri were removed using an aseptic

surgical technique under ketamine/lidocaine anesthesia. Uteri were immediately frozen in liquid N_2 and stored at $-80^{\circ}C$ until assay.

Radioimmunoassay

Circulating levels of estradiol were measured using a commercially available radioimmunoassay (RIA) kit from ICN Biomedicals, INC., (Carson, CA) (Abraham, 1975). 6-keto-estradiol-17ß-6-Oxime-BSA was used as the antigen to generate the antiserum in rabbits. Cross reactivities of the antibody with other estrogens were as follows: estrone, 20%; estriol, 1.51%; estradiol-17α, 0.68%. The antiserum was diluted in MES/gelatin buffer to bind 40-55% of the [^{125}I]-estradiol derivative. Estradiol concentrations used to generate the standard curve ranged from 0-3000 pg/ml.

The assay mixture contained 50 µl of undiluted serum and 500 µl of [^{125}I]-E_2. To this, 500 µl of anti-E_2 was added to all tubes except the non-specific binding (NSB) tube. Incubations were at $37^{\circ}C$ for 90 min. After incubation, 500 µl of the precipitating solution (goat anti rabbit gamma globulin) was added to each tube, followed by centrifugation at 1000 x g for 20 min. The supernatant was aspirated and the precipitant remaining was counted in a gamma scintillation counter calibrated for ^{125}I. Results were calculated using a standard RIA data reduction system.

Preparation of Subcellular Fractions

Individual homogenates of each uterus were prepared by two 15 s bursts at setting 6 with a Brinkman Polytron (Brinkman Instruments, Westbury, NY) in 10 mM Tris-HCl, 1.5 mM EDTA, 1 mM dithiothreitol (DTT) and 10% glycerol, pH 7.4 (TEDG buffer), utilizing 0.1 gram of tissue per 10 ml buffer. Homogenates were centrifuged at 800 x g for 20 min. in a Beckman J-21 centrifuge. The 800 x g pellet obtained was designated as crude nuclear pellet. The supernatants were then centrifuged at 105,000 x g for 1 h in a Beckman L5-65 ultracentrifuge. The supernatant from the last step was designated as cytosol and was used in subsequent assays.

Nuclear Exchange Assay

Specific nuclear estradiol binding sites were assayed by the method described by Anderson et al. (1972). The crude nuclear pellets were washed three times with three ml of the TEDG buffer and each wash was followed by centrifugation at 800 x g for 10 min. The washed pellet was suspended in TEDG buffer to a concentration equivalent to 250 mg protein per ml and gently re-homogenized. Aliquots (0.25 ml) of this suspension were dispensed into two series of 6 tubes labeled A and B containing 0.05 ml of TEDG buffer. Series A contained various concentrations

(0.75-5.0 nM) of [^{3}H]-E_2 and was used to determine the total amount of [^{3}H]-E_2 exchanged. Series B contained the same concentration of [^{3}H]-E_2 as in Series A plus a 100-fold excess of diethylstilbestrol (DES). The B series was used to determine the specific [^{3}H]-E_2 exchange. The crude nuclear assay mixtures were incubated with gentle shaking at 37°C for 30 min. After incubation, 1 ml of ice cold TEDG buffer was added to the assay tubes, followed by centrifugation at 800 x g for 10 min. Pellets were washed three times with TEDG buffer and extracted with 1 ml 100% ethanol followed by centrifugation at 800 x g for 10 min. The ethanol extract was added to 5 ml of scintillation cocktail (Safety Solve, Research Products Inc. Mt. Prospect IL).

Cytosol Assay:

The cytosol fraction was assayed according to the method of William and Gorski (1975). Aliquots (0.25 ml) of this suspension were dispensed into two series of 6 tubes labeled A and B containing 0.05 ml of TEDG buffer. Series A contained various concentrations (0.75-5.0 nM) of [^{3}H]-E_2 and was used to determine the total amount of [^{3}H]-E_2 exchanged. Series B contained the same concentration of [^{3}H]-E_2 as in Series A plus a 100-fold excess of diethylstilbestrol (DES). The series B was used to determine the specific [^{3}H]-E_2 exchange. Incubation tubes were placed in an ice water bath for 5-10 min.

Aliquots 0.25 ml of 60% (v/v) hydroxylapatite (HAP)-TEDG buffer suspensions were added to each tube and were left on ice for 15 min. with vortexing at five minute intervals. The assay tubes were centrifuged at 800 x g for 10 min. HAP pellets were washed four times by suspension in 1 ml aliquots of the homogenization buffer. This was followed by centrifugation at 800 x g for 6 min. After the last wash, 1 ml of 100% ethanol was added to each tube. The tubes were then placed in a 30°C water bath for 10 min. The tubes were vortexed followed by centrifugation at 800 x g for 10 min. Finally, the ethanolic extracts were transferred to scintillation vials.

Statistical Analysis

The calculation of dissociation constants and [^{3}H]-E_2 binding capacities were determined according to the method of Scatchard (Scatchard, 1949) after subtraction of non-specific binding. The best fit was determined using a computerized least square analysis. Data groups were compared using a Student's t test.

In Vivo Metabolic Studies

Six young female squirrel monkeys (Saimiri sciureus) weighing 0.6-0.7 kg, were quarantined for approximately six weeks during which time parasites were eliminated. The animals were subsequently housed in pairs in a temperature-

and-light controlled room for several weeks before the studies were begun. Animals were transferred to metabolic cages and radiolabeled estradiol-17ß (75 µCi in 250 µl saline solution) was administered by femoral injection. The urine and feces excreted on each of the 4 succeeding 24 h intervals were collected.

Separation of Conjugates from the Urine

The preliminary separation of tritium-labeled free steroids and estrogen conjugates from the urine was performed using Baker's octadecyl solid phase extraction system (J.T.Baker, Phillipsburg, NJ). Briefly, each urine collection was loaded onto the column. Estrogens were eluted by 2 successive applications of 3 ml methanol (Morris et al., 1982). Radioactive content of each was determined by taking an aliquot of each for scintillation spectrometry.

Extraction of Radioactivity from the Feces

Fecal collections were extracted with ethanol using the Soxhlet extraction apparatus. Extraction procedures were carried out for 24 h. Ethanolic extracts were subsequently evaporated followed by resuspension in 3 ml distilled water. Aliquots of 0.5 ml were taken for analysis by scintillation spectrometry.

DEAE-Sephadex Chromatography

The ethanolic extracts of each 24-h fecal collection was evaporated to dryness, redissolved in 3 ml of water and chromatographed on a (60 x 1 cm) DEAE-Sephadex A-25 in a linear NaCl gradient (0.0-2.0 M)(Hobkirk et al., 1969). Ten ml fractions were collected, and 0.5 ml aliquots of each were taken for scintillation spectrometry.

Thin Layer Chromatography

Following organic partitioning, aliquots of each sample were chromatographed on thin layer plates (silica gel) in the following systems: System I benzene/ethyl acetate (6:4); System II, 10% NH_4OH saturated n-butanol. Unlabeled reference standards were run in separate channels. Steroids were visualized on the chromatographic plates by spraying with 2% (v/v) H_2SO_4 in methanol and then heating the plates to 100°C for 5-10 min. Chromatographic mobilities of radiolabeled estrogens were determined by cutting chromatographic plates into 1 x 3 cm strips and placing them into scintillation vials for counting. The R_f values for the reference standards E_1, E_2, and E_3 in system I were 0.79, 0.61, and 0.12, respectively. The R_f values for conjugated estrogen standard $E_2$17ßS, $E_2$3S, and $E_2$3,17S in system II were 0.61, 0.61, and 0.56, respectively.

Identification of Unconjugated Estrogens

Aliquots of radioactive material eluted from the DEAE-Sephadex columns were extracted 3 times with ether. The ether extracts were evaporated, and the extract was chromatographed in system I.

Enzyme Hydrolysis

Further identification of radioactive material eluted from DEAE-Sephadex eluant fractions was established by enzyme hydrolysis. Aliquots of pooled radioactive material eluting between fractions 25-50, were adjusted to pH 5.0 with sodium acetate buffer. Aliquots were then incubated with ß-glucuronidase with 100 units per 10 ml. Radioactive material eluting in fractions 52-80 was incubated with phenolsulfatase (12 mg/5 mL) in 0.10 M sodium acetate buffer, pH 6.0. All incubations were carried out for 24 h at 37°C. Incubation mixtures were then extracted with ether. The free estrogens extractable by ether were identified by TLC as described previously.

Characterization of Cytosolic E_2-17ß Sulfotransferase Activity

Frozen livers (20 g) were homogenized in one volume of ice cold 10 mM Tris-HCl buffer pH 7.4, supplemented with 3 mM ß mercaptoethanol, 0.25 mM sucrose. This and all other preparative procedures were carried out at 0-4°C. The

homogenates were centrifuged for 20 min at 800 x g. The supernatant generated was re-centrifuged for 1.5 h at 140,000 x g in a Beckman L-8M 70 ultracentrifuge. The resulting supernatant, designated cytosol, was the source of enzyme for all studies, unless otherwise noted.

Standard Sulfotransferase Assay

The assay used in this investigation was adapted from the published method described by Adams and coworkers (1974). The E_2-17ß sulfotransferase was assayed in a mixture that contained 175 µl cytosol, (containing 730 µg protein), 0.2 mM PAPS, [2,4,6,7 ^{3}H]-E_2 (10^6 DPM), 0.008 mM E_2 and 2 mM $MgCl_2$ in a total volume of 250 µl at a pH of 7.4. An assay blank contained all the components of the assay mixture except for PAPS. After incubation at 37°C for 20 min, the reaction was terminated by the addition of 1 ml cold methanol. Assay incubation mixtures were stored overnight at -20°C to precipitate protein. Following cold precipitation of proteins, the assay mixture was centrifuged at 1000 x g and the supernatant decanted. Identification of estradiol-17ß sulfation product was established by chromatography on DEAE-Sephadex columns (0.9 x 30 cm) with reference [^{3}H]-$E_2$17S.

Partial Purification of E_2-17ß Sulfotransferase

The supernatant fraction obtained from squirrel monkey liver homogenate was submitted to a precipitation procedure using ammonium sulfate [$(NH_4)_2SO_4$] in solid form to arrive at 50% saturation. The precipitation was allowed to proceed for 1 h at 4°C. The precipitate recovered by centrifugation (15,000 x g) was dissolved in a 50 mM Tris-HCl, 25 mM sucrose, 3 mM ß mercaptoethanol, pH 7.9 buffer The same buffer was used to equilibrate a 1 x 20 cm column of DEAE-cellulose. Prior to loading onto the column, the resuspended $(NH_4)_2SO_4$ precipitant was desalted on a Sephadex G-25 column. The eluant was concentrated and was applied to the column. The column was eluted at 1 ml/min with a linear KCl gradient (0.0-0.5 M). Three ml fractions were collected. Absorbance was monitored at 280 nm and each fraction was assayed for estradiol 17ß-sulfotransferase activity. The more active fractions were pooled and concentrated with Amicon B-15 membranes. The pH of the extract was then adjusted to 7.9 with 5 N HCl prior to chromatography on a 5 ml column (1 cm diameter) of PAP-agarose gel, equilibrated in 50 mM Tris-HCl, 25 mM sucrose, 3 mM ß mercaptoethanol, pH 7.9. The flow rate was set to 1 ml/min. The affinity column was washed with five column volumes of the same buffer and 3.0 ml fractions were collected. Elution of the adsorbed protein was achieved by the addition of 0.6 mM PAPS to the washing buffer. After

adding PAPS, the fractions collected were 0.7 ml in volume. The protein concentration was quantified by monitoring at 280 nm.

CHAPTER IV

EXPERIMENTAL RESULTS

Determination of Plasma Estradiol Levels

As an index of ovarian cyclicity, plasma E_2 levels were determined prior to estrogen treatment. The mean plasma E_2 concentrations for the six monkeys are presented in Table 1. In the six squirrel monkeys, the plasma E_2 levels ranged from 9.01 to 26.91 pg/ml with a mean plasma E_2 concentration of 17.59±7.85 pg/ml.

Receptor Binding Parameters

Uterine receptor binding parameters in control and E_2-treated squirrel monkeys were determined as described by Anderson (Anderson et al., 1972). As shown in Table 2, cytosolic fractions prepared from vehicle-injected animals exhibited no specific binding of $[^3H]$-E_2. Saturable binding sites for E_2 were detected in the crude nuclear pellets. Analysis of the nuclear binding sites by Scatchard plots revealed a mean binding capacity of 35.0±21.0 pmol/L with a dissociation constant of 6.29±1.7 nM (Figure 1). In those animals primed with estradiol for two consecutive days, cytosolic receptor concentrations

Table 1

Plasma Estradiol Levels in Young Female Squirrel Monkeys

Squirrel Monkey#	Estradiol (pg/ ml)
1	9.18±2.18
2	9.01±0.82
3	25.9±0.37
4	22.4±3.18
5	10.9±1.09
6	12.9±3.30

Each value represent the mean concentration
n=2

Table 2

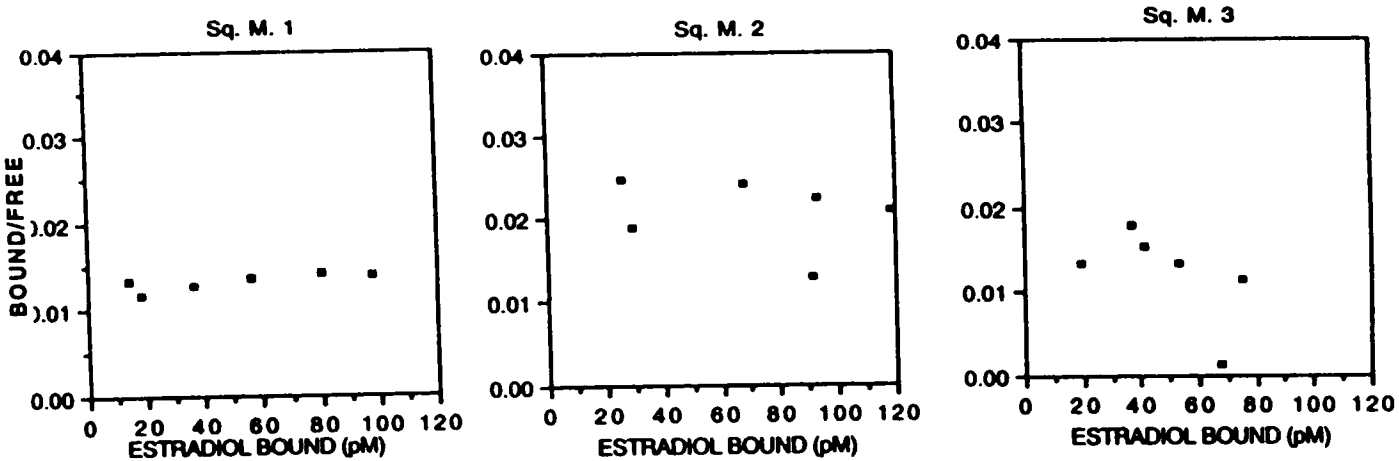
Estrogen Receptor Binding Characteristics in Control and Estrogen Treated Squirrel Monkeys

	CYTOSOL		NUCLEAR	
Control	Rt(pmol/l)	Kd(nM)	Rt (pmol/l)	Kd (nM)
1	ND	ND	14.0	4.83
2	ND	ND	35.0	5.83
3	ND	ND	56.0	8.20
Mean± SD			35.0±21.0	6.29±1.73
E2 Treated				
4	245	2.89	68.0	4.20
5	230	2.98	42.0	7.63
6	210	2.53	30.0	4.42
Mean±SD	288±17.6	2.80±0.24	46.0±19.4	5.42±1.92

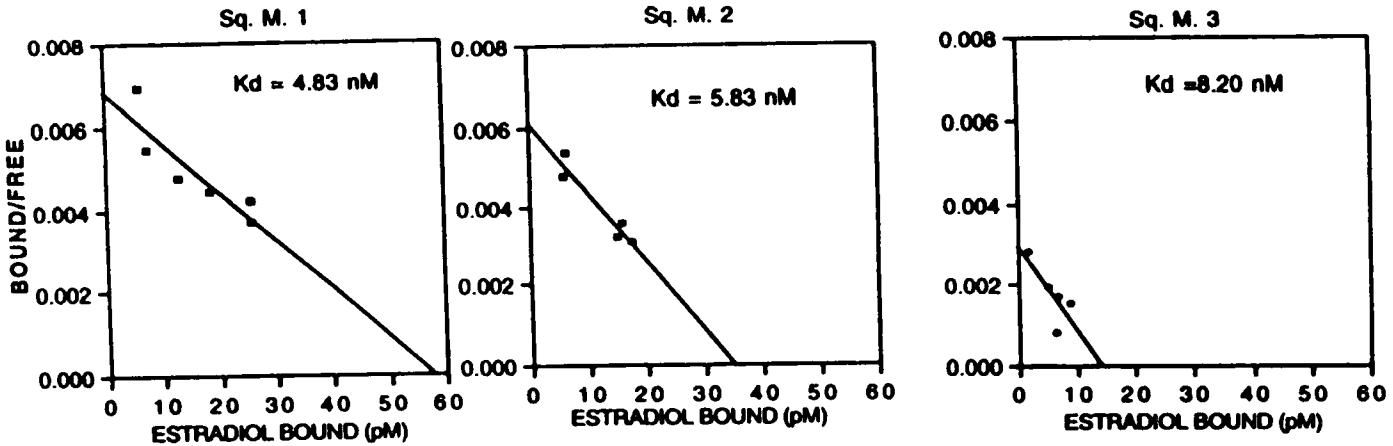
ND indicates no detection of specific binding and Rt denotes total receptor

Figure 1. Scatchard Plots of [^{3}H]-Estradiol Binding in Uterine Tissue of Untreated Squirrel Monkeys. Aliquots of each uterine subcellular fraction were incubated with various concentrations of [^{3}H]-estradiol (0.75-5.0 nM) in the presence and absence of 100-fold excess diethylstilbestrol (DES) for 30 min at 37°C.

CYTOSOL



NUCLEUS



were dramatically increased (Figure 2). In these monkeys, an average receptor concentration of 228±17.6 pmol/l was obtained with an average dissociation constant of 2.80±0.24 nM. The concentration of ER in crude nuclear pellets of treated monkeys was found to be 46.0± 19.4 pmol/l with a dissociation constant of 5.42±1.92 nM.

Distribution of Radioactivity in Urine and Feces

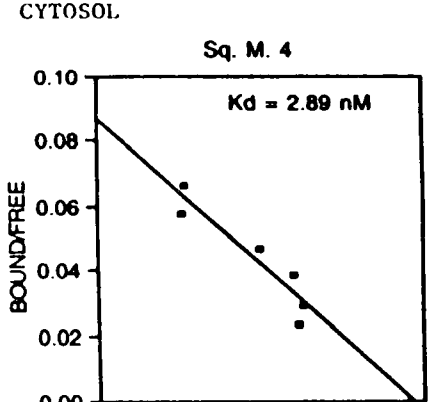
Figure 3 shows the distribution of radioactivity in urine and feces following femoral injection of [^{3}H]-E_2. As shown in the figure, approximately 75% of the administered radioactive dose was recovered after four days post-injection. On day 1, 65% of the radioactivity was excreted, 60% accountable for in the feces and 4% in the urine. On day 2, total excretion of radiolabeled material dropped to 5%, almost all of which was recovered in the feces. Excretion of radioactivity had decreased to about 3% on day 3 and was down to only 2% by the fourth day. Excretion of radiolabeled compounds after the fourth day was negligible.

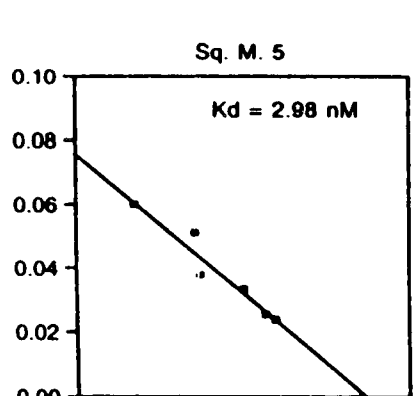
Identification of [^{3}H]-Labeled Estrogen Metabolites in Urine

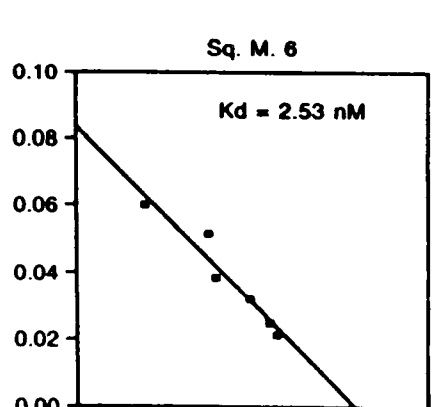
To identify the individual urinary metabolites excreted following injection of [^{3}H]-E_2, urine samples were

Figure 2. Scatchard Plots of [^{3}H]-Estradiol Binding in Uterine Cytosol and Crude Nuclear Pellets of Estradiol-primed Squirrel Monkeys.
Aliquots of 105,000 x g supernatant prepared from uterine tissue were incubated with various concentrations of [^{3}H]-estradiol in the presence and absence of 100-fold excess diethyl-stilbestrol for 30 minutes at 37°C.

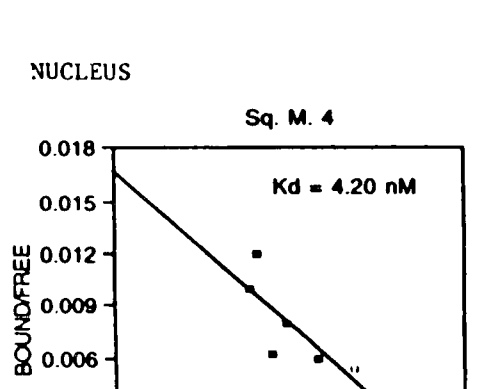
CYTOSOL

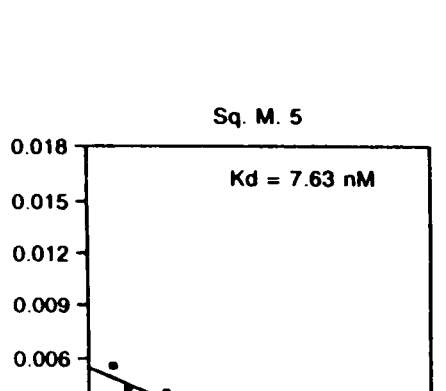






NUCLEUS





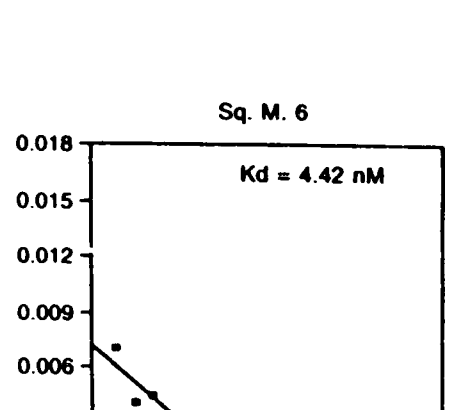
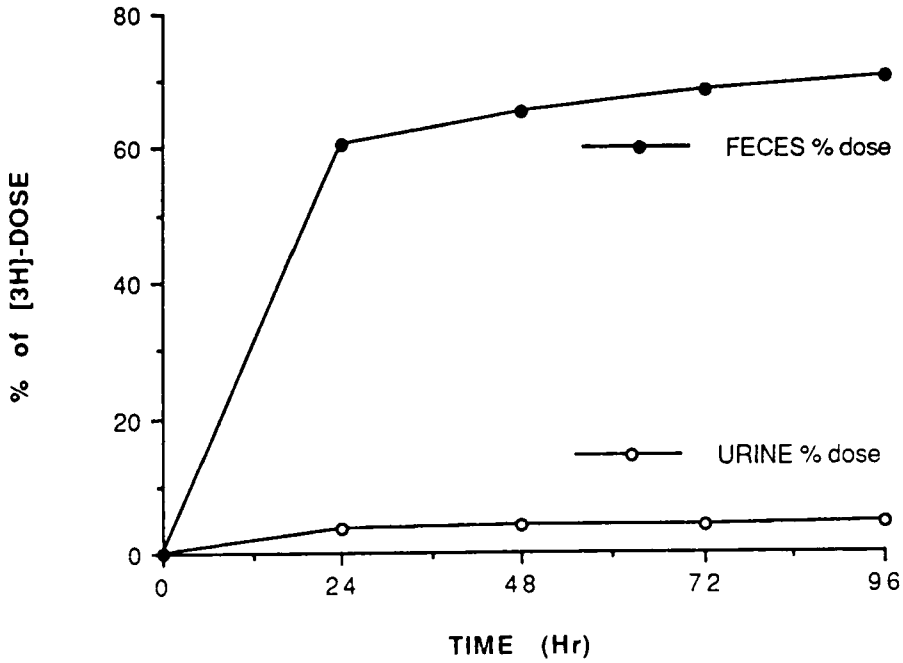


Figure 3. Distribution of Radioactivity in Urine and Feces in Squirrel Monkey Following Intravenous Administration of [^{3}H]-Estradiol.

Daily urine and fecal samples were collected for a duration of 96 hours following intravenous administration of [^{3}H]-E_2. Total radioactive recovery was determined for each fraction and is expressed as the percent of total administered dose.


80
60
40
20
0
% of [3H]-DOSE
FECES % dose
URINE % dose
0
24
48
72
96
TIME (Hr)

fractionated by DEAE-Sephadex chromatography. Seven major peaks were obtained (Figure 4). Peaks were labeled in order of their elution from the column. On the basis of chromatographic profiles, following elution from a DEAE-Sephadex column, peaks I and II were tentatively identified as free, unconjugated estrogens. Glucosiduronate conjugates eluted between fractions 25-40. Peaks V and VI, eluting between fractions 50-80 corresponded to monosulfates. Further elution with high NaCl (0.8-2.0 M) revealed a seventh peak, tentatively identified as a diconjugate, eluting in fractions 140-155.

Identification of Peaks I and II

The identities of urinary estrogens were established via TLC and enzyme hydrolysis as shown in Table 3. Organic partitioning of radioactivity from peaks I and II resulted in about 98% of the radioactivity extractable by ether. Thin layer chromatography of ether extracts from peak I and II in system I yielded R_f values of 0.79 and 0.61, respectively. These values were identical to those of authentic E_1 and E_2 reference standards.

Identification of Peaks III and IV

ß-glucuronidase hydrolysis of aliquots of radioactivity from peaks III and IV, rendered 97% of the radioactivity extractable by ether. The chromatographic

Figure 4. Representative DEAE-Sephadex Chromatographic of 24-Hour Urinary Sample of Squirrel Monkey Urine samples were collected and concentrated using a Baker's C_{18} solid phase extraction system. Concentrated urine was then loaded onto a DEAE-Sephadex column (1 x 50 cm). The column was eluted with a linear NaCl gradient (0.0-2.0 M) with a total volume of 1000 ml. Ten ml fractions were collected and 1.0 ml aliquots of each fraction were analyzed by scintillation spectrometry.

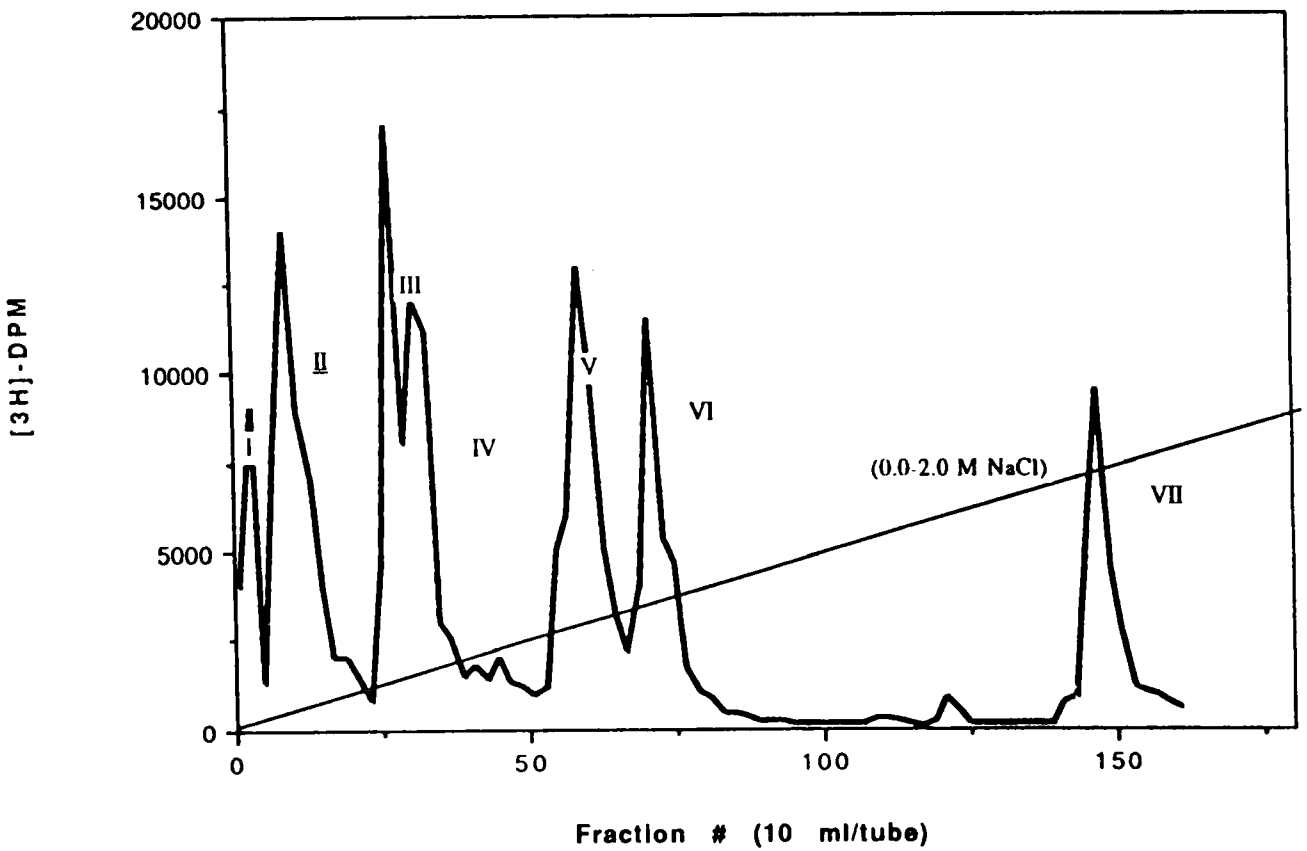

20000
15000
10000
5000
0
[3H]-DPM
I
II
III
IV
V
VI
VII
(0.0-2.0 M NaCl)
0
50
100
150
Fraction # (10 ml/tube)

Table 3

Identification of [^{3}H]-Estrogen Metabolites in Squirrel Monkey Urine Following Elution from DEAE-Sephadex Column

PEAK	Total DPM	Enzyme Hydrolysis	Ether Extraction Organic %DPM	Ether Extraction Aqueous %DPM	TLC *Solv.I Rf	TLC Solv. II Rf
I	3.3 x10 ^{4}	--	97.0	2.89	0.79	--
II	3.2 x10^4	--	98.1	2.11	0.61	--
III	2.5 x10 ^{4}	ß/ase	96.8	3.67	0.79	--
IV	1.5 x10 ^{4}	ß/ase	97.2	3.01	0.61	--
V	2.6 x10 ^{4}	s/ase	99.1	1.00	0.79	--
VI	2.2 x10 ^{4}	s/ase	0.01	98.2	0.01	0.61
		ß/ase	0.98	99.8	0.01	0.61
VII	1.7 x10 ^{4}	--	0.01	98.6	0.01	0.56
		s/ase	0.01	99.0	0.01	0.61

* solvent system I: benzene/ethyl acetate (6:4)
solvent sytem II: 10% NH_4OH-saturated n-butanol

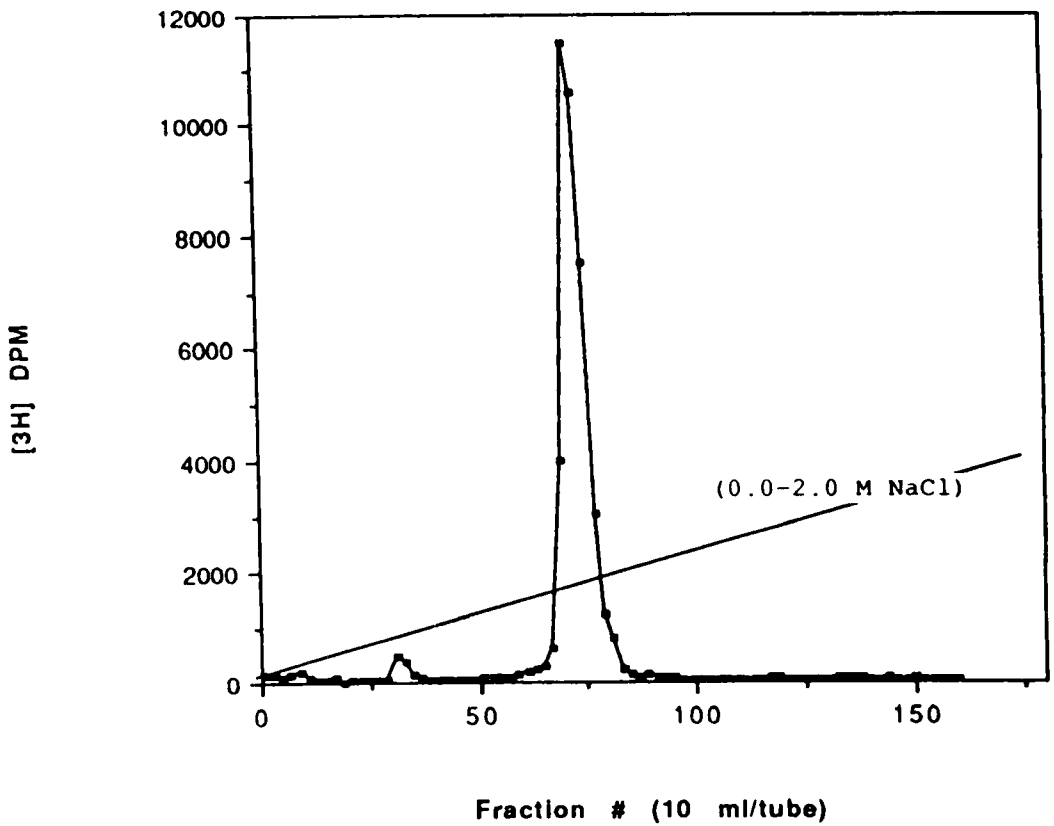
mobility in system I of the ether extract derived from peak III was 0.79 which is identical to that of reference E_1 standard. When chromatographed on thin layer plates in system I, most of the radioactivity from peak IV migrated as E_2, with an R_f value of 0.61. As a result of these analyses, peaks III and IV were identified as estrone glucosiduronate (E_1G) and estradiol glucosiduronate (E_2G), respectively.

Identification of Peaks V and VI

After enzyme hydrolysis of Peak V with phenolsulfatase, 99% of the radiolabeled material was recovered in the ether extract. TLC (system I) of ether extracts showed that the labeled material migrated with a R_f value of 0.79, identical to that of authentic estrone. Thus, peak V was identified as E_1S. Incubation of aliquots of peak V with phenolsulfatase, resulted in the extraction of only 2% of the radioactivity by ether. Further identification of the radioactive material remaining in the aqueous fraction was achieved by re-chromatography on DEAE-Sephadex with reference [^{3}H]-$E_2$17S. The chromatographic profile is shown in Figure 5. The elution pattern was comparable to that of reference [^{3}H]-$E_2$17S. TLC of the sample peak in system II gave a simple radioactive chromatographic profile with an R_f of 0.61, identical to standard $E_2$17S; therefore, peak VI was identified as $E_2$17S.

Figure 5. DEAE-Sephadex Chromatogram of Enzyme Extract from Peak V.

After incubation with phenolsulfatase, aliquots of peak V were subjected to ether extraction. Radioactivity recovered in the aqueous phase (97%) was applied to a DEAE-Sephadex column (60 x 0.9 cm; 0.0-2.0 M NaCl) with reference [^{3}H]-$E_2$17S.


12000
10000
8000
6000
4000
2000
0
[3H] DPM
(0.0-2.0 M NaCl)
0
50
100
150
Fraction # (10 ml/tube)

Identification of Peak VII

Thin layer chromatography of aliquots of Peak VII in system II corresponded in polarity with that of an estrogen diconjugate. Following incubation with phenolsulfatase and ß-glucuronidase, the majority of the radioactivity remained in the aqueous phase. Chromatographic mobilities of radioactivity recovered in the aqueous phase in system II revealed that the phenolsulfatase-incubated fraction now behaved as a monosulfate (R_f=0.61) whereas that incubated with ß-glucuronidase still corresponded to that of the diconjugate (R_f=0.56). These results showed that at least one of conjugate groups of the diconjugate was a sulfate ester. Re-chromatography of the phenolsulfatase-hydrolyzed fraction on DEAE-Sephadex with reference [^{3}H]-$E_2$17S resulted in the elution of a single peak. Peak VII was identified as $E_2$3,17-disulfate.

The relative distribution of the radioactivity in the individual estrogen metabolites over 4 days is shown in Table 4. Excretion of unconjugated estrogen was relatively high on day 1 representing 30.5% of the total urinary radioactivity. By day 2, urinary excretion of unconjugated estrogens dropped to only 3%. On days 3 and 4, unconjugated estrogen represented small, but detectable amounts of radioactivity. The glucosiduronate fraction, comprised almost entirely of E_1G and E_2G, represented

Table 4

Excretion of Urinary Estrogen Metabolites Following Intravenous Administration of [^{3}H]-Estradiol

% of Total Urinary [^{3}H]

HR	FREE	E_1G	E_2G	E_1S	E_217S	E_2diS
24	30.5	11.3	7.00	12.0	10.0	8.00
48	3.00	2.00	0.60	0.50	0.80	0.50
72	0.50	1.00	0.40	0.50	0.30	0.40
96	0.40	0.90	0.40	0.40	0.50	0.40
Total%	34.4	15.2	8.40	13.4	11.6	9.30

% of Total Dose

HR	FREE	E_1G	E_2G	E_1S	E_217S	E_2diS
24	1.29	0.48	0.30	0.51	0.43	0.34
48	0.13	0.09	0.03	0.02	0.04	0.02
72	0.02	0.04	0.02	0.02	0.01	0.02
96	0.02	0.04	0.02	0.02	0.02	0.02
Total%	1.46	0.65	0.37	0.57	0.50	0.40

nearly 24% of total urinary estrogens. Excretion of E_1G exceeded E_2G during the first 24 h, 11.3% versus 7%. On days 2-4, this excretory pattern of glucuronides was repeated with only fractional differences in the amount of E_1G and E_2G excreted. Sulfated estrogens represented 30% of the total urinary radioactivity. Over the 4 days, E_1S, E_217S and $E_23,17$-diS accounted for 13%, 12%, and 9%, respectively. Figure 6 represents a cumulative excretion of labeled metabolites in the urine.

Identification of [3H]-Labeled Metabolites in Feces

Initial separation of fecal metabolites was carried out by anion exchange (DEAE-Sephadex A-25) chromatography. Figure 7 shows a representative chromatogram of estrogen metabolites eluted from DEAE-Sephadex. Radiolabeled material, representing unconjugated estrogens, eluted early followed sequentially by the glucosiduronate fractions, sulfoconjugates, and finally at increased NaCl, a diconjugate fraction.

Identification of individual peaks was established as shown in Table 5. Ether extracts of peaks I and II chromatographed on thin layer plates in system I resulted in chromatographic mobilities of 0.79 and 0.61 corresponding to that of authentic E_1 and E_2. Following incubation of a small portion of peak III with ß-

Figure 6. Cumulative Excretion of Tritium-labeled Estrogen Metabolites in Urine of Squirrel Monkey Following Intravenous Administration of [^{3}H]-Estradiol

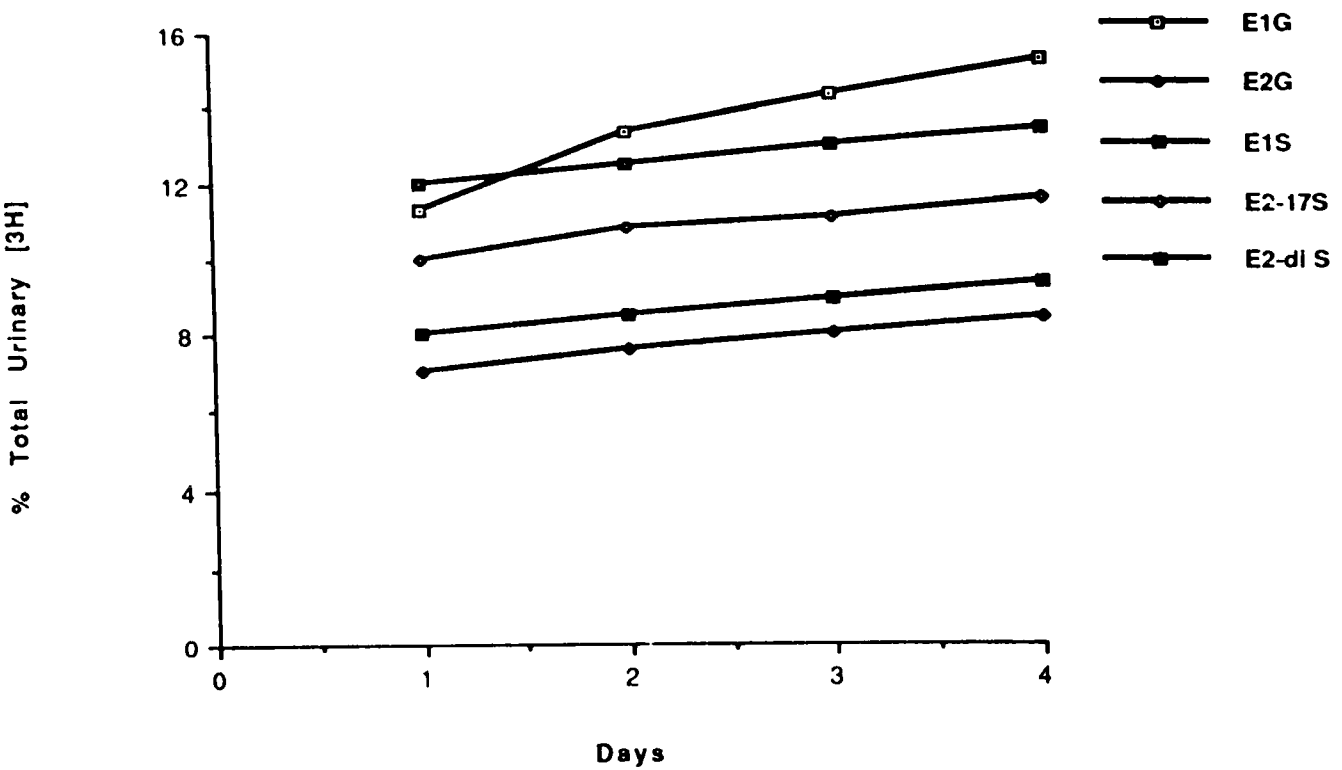

% Total Urinary [3H]
16
12
8
4
0
0
1
2
3
4
Days
E1G
E2G
E1S
E2-17S
E2-di S

Figure 7. Representative DEAE-Sephadex Chromatogram of 24-Hour Fecal Extract of Squirrel Monkey. Fecal samples were collected and concentrated using Soxhlet extraction system. Concentrated fecal extracts were then loaded onto a DEAE-Sephadex column (1 x 50 cm). The column was eluted with a linear NaCl gradient (0.0-2.0 M) in a total volume of 1000 ml. Ten ml fractions were collected and 1.0 ml aliquots of each fraction were analyzed by scintillation spectrometry.

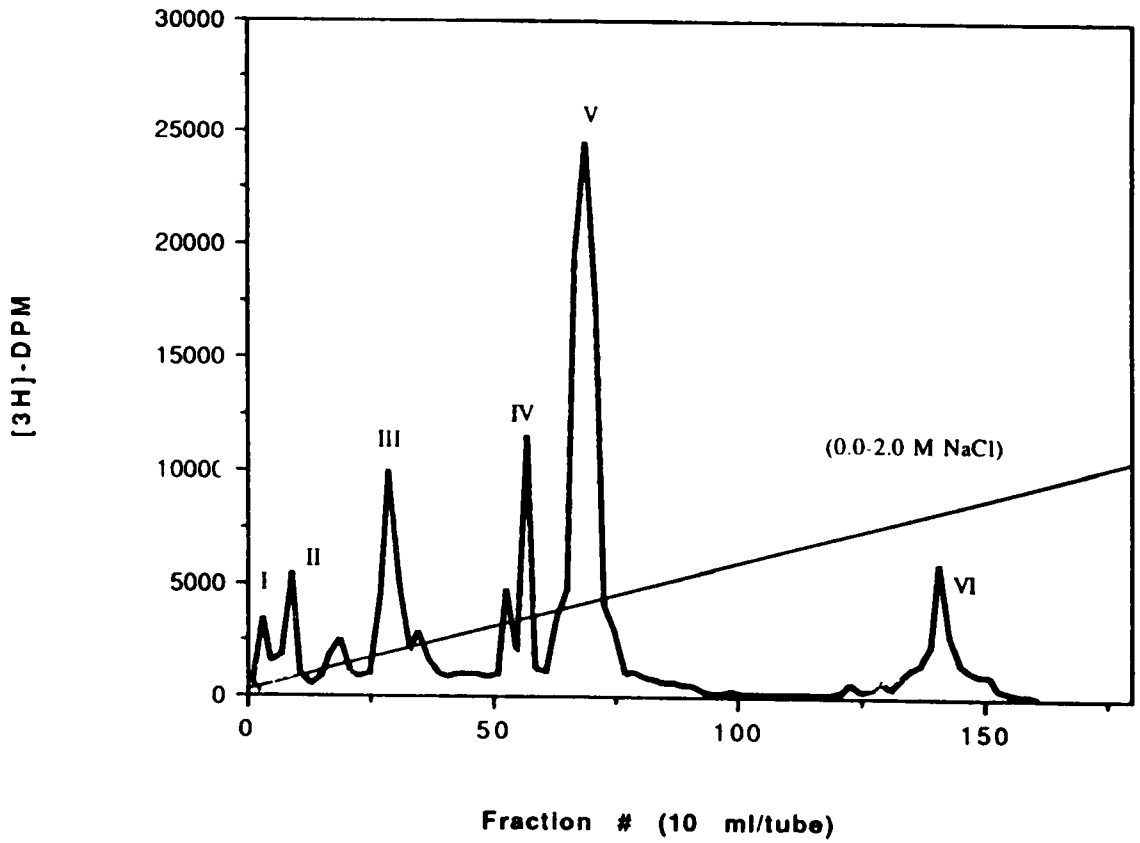

30000
25000
20000
15000
10000
5000
0
0
50
100
150
I
II
III
IV
V
VI
(0.0-2.0 M NaCl)
[3H]-DPM
Fraction # (10 ml/tube)

Table 5

Peak Identification of [^{3}H]-Estrogen Metabolites in Squirrel Monkey Feces Following Elution from DEAE-Sephadex

PEAK	Total DPM	Enzyme Hydrolysis	Ether Extraction Organic %DPM	Ether Extraction Aqueous %DPM	TLC Solv. I R_f	TLC SolvII R_f
I	$4.6x10^4$	--	98.8	2.36	0.79	--
II	$5.1x10^4$	--	97.5	3.43	0.61	--
III	$2.0x10^4$	ß/ase	97.9	2.74	0.79	--
IV	$8.0x10^4$	s/ase	95.0	2.83	0.79	--
V	$6.7x10^5$	s/ase	0.03	99.1	0.01	0.61
		ß/ase	0.01	99.0	0.01	0.61
VI	$1.1x10^5$	--	0.02	98.7	0.01	0.56
		s/ase	0.05	98.8	0.01	0.61

*solvent system I: Benzene/ethyl acetate (6:4)
solvent system II: 10% NH_4OH -saturate n-butanol

glucuronidase, 97% of the radioactivity was recovered as free steroid. Subsequent TLC in System I indicated that peak III was E_1G. Hydrolysis by phenolsulfatase liberated virtually all of the radioactivity from peak IV as free steroid, which yielded a chromatographic mobility of 0.79. A major portion of the radioactivity (98%) of peak V was not hydrolyzable by incubation with phenolsulfatase. Analysis of the aqueous fraction by TLC in system II gave an R_f value of 0.61, also indicative of conjugated estrogen metabolite. Chromatography of a portion of this aqueous fraction on DEAE-Sephadex along with standard [^{3}H]-$E_2$17S resulted in the elution of a single peak. Incubation of portions of peak VI with either ß-glucuronidase or phenolsulfatase did not yield ether extractable radioactive material. Chromatographic mobilities of the enzymatic extracts in system II demonstrated that the phenolsulfatase incubation rendered radioactive material that now migrated as a less polar component. When the enzymatic extract was re-chromatographed on DEAE-Sephadex, the radiolabeled material eluted as a single peak. TLC of an aliquot in system II gave a R_f value of 0.61, corresponding to $E_2$17S.

Estrogen metabolites recovered in fecal extracts are presented in Table 6 along with the relative amounts of radiolabeled material contained in each. The unconjugated fractions were comprised primarily of estrone and estradiol. On day 1, unconjugated estrogens represented 7%

Table 6

Excretion of Fecal Estrogen Metabolites Following Intravenous Administration of [^{3}H]-Estradiol

% OF TOTAL FECAL [^{3}H]

	HR	FREE	E_1G	E_1S	E_23S	E_217S	E_2diS
	24	7.30	15.0	6.00	--	50.0	8.00
	48	0.80	2.00	0.50	--	3.10	1.00
	72	--	1.00	0.30	0.20	1.00	0.50
	96	--	1.00	0.50	0.20	1.00	0.30
Total	%	8.10	19.0	7.30	0.40	55.1	9.80

% of TOTAL DOSE

	HR	FREE	E_1G	E_1S	E_23S	E_217S	E_2diS
	24	5.14	10.6	0.35	--	35.2	5.63
	48	0.56	1.40	0.04	--	2.18	0.70
	72	--	0.70	0.02	0.01	0.71	0.03
	96	--	0.70	0.03	0.01	0.70	0.02
Total	%	5.70	13.4	0.44	0.02	38.8	6.39

of the total radioactivity recovered in the feces but decreased to less than 1% by day 2. Fecal excretion of unconjugated estrogens was undetectable on days 3 and 4. The principal estrogen excreted in the glucosiduronate conjugate fraction was E_1G. Over four days, estrone glucosiduronate accounted for about 20% of total fecal radioactivity with 21% excreted on day 1. The major monosulfates identified in feces were E_1S and E_217S. Minute amounts of radioactivity corresponding to E_23S were detected on days 3 and 4. E_217S was the major estrogen metabolite accounting for about 50% of the fecal radioactivity on day 1, increasing to a cumulative total of 55 % by day 4. On day 1, E_1S represented 6% of the total fecal radioactivity. During the following days, its excretion in feces showed some minor fluctuations, but no significant changes in excretory pattern. The diconjugate fraction was identified as $E_23,17$-diS. Figure 8 represents a cumulative excretion pattern of radioactivity in the feces over the four days.

Optimum Assay Conditions for Estradiol-17ß-sulfotransferase

Estradiol-17ß sulfation as a function of concentration of protein obtained from squirrel monkey liver cytosol was determined. The results are shown in Figure 9. In a one hour incubation with 0.008 mM E_2, the amount of E_217S formed was linear up to protein concentration of 150-820

Figure 8. Cumulative Excretion of Tritium-labeled Estrogen Metabolites in Feces of Squirrel Monkey Following Intravenous Administration of [^{3}H]-Estradiol

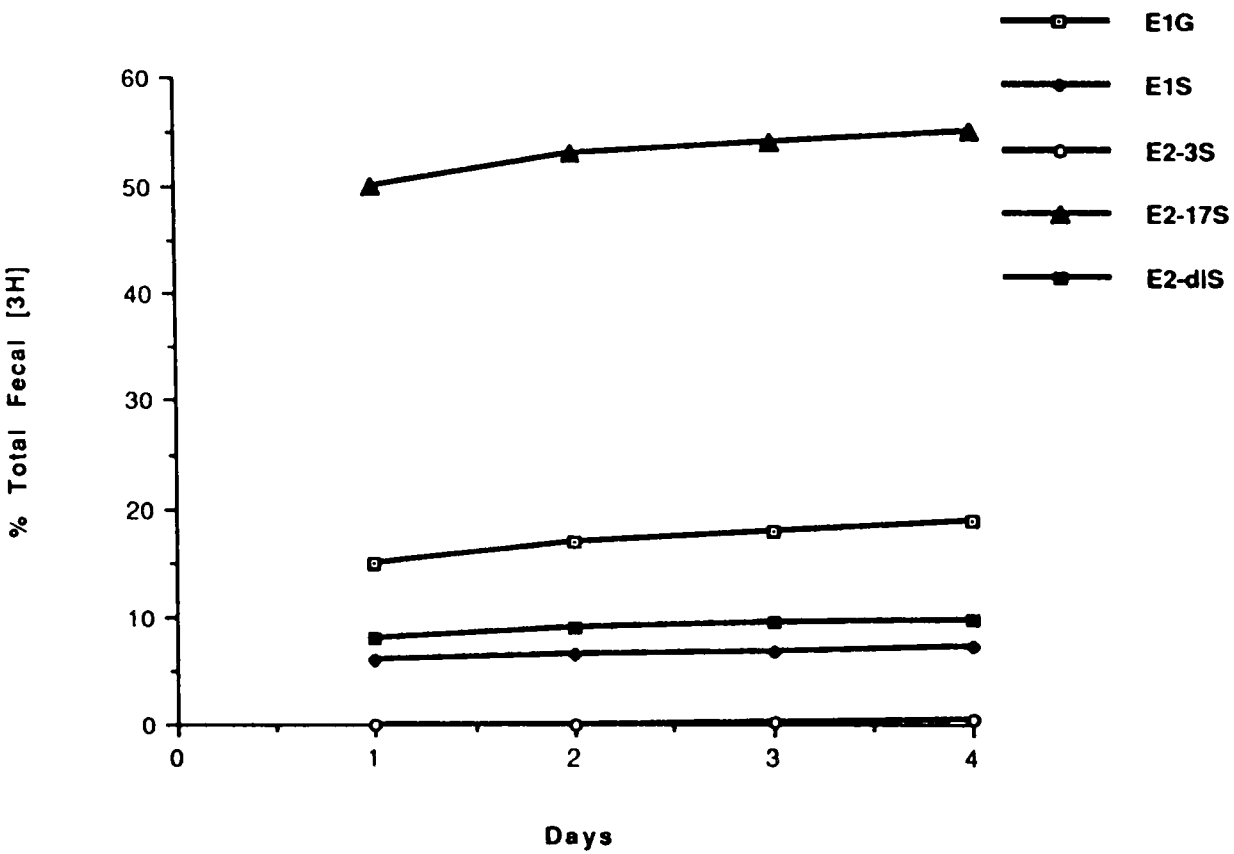
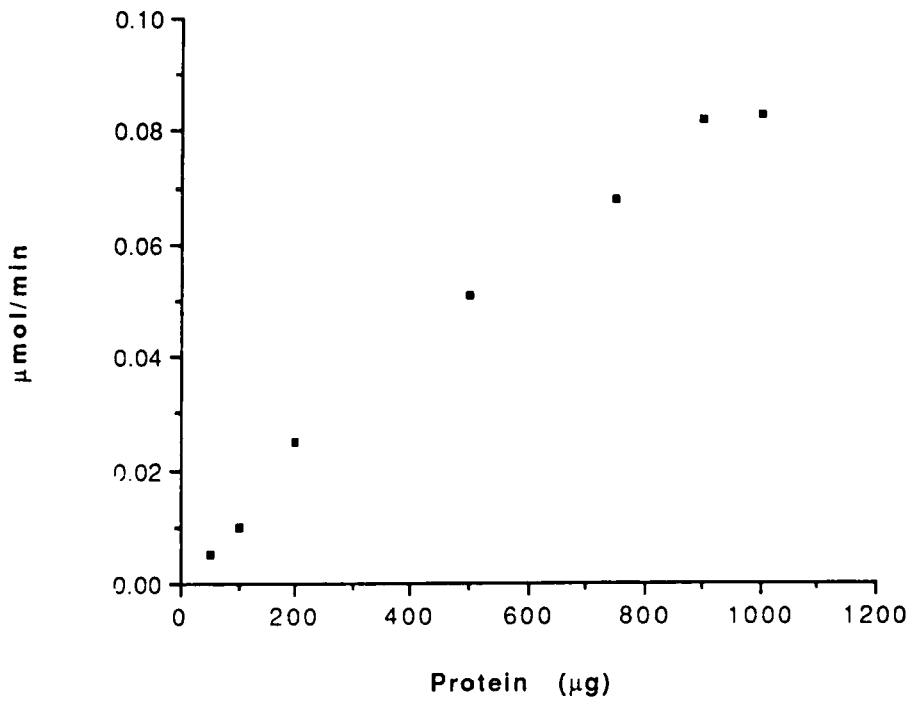

E1G
E1S
E2-3S
E2-17S
E2-dlS
% Total Fecal [3H]
60
50
40
30
20
10
0
0
1
2
3
4
Days

Figure 9. Effect of Cytosol Concentration on E_2-17ß Sulfotransferase Activity.

Incubations were carried out for 20 min. at 37°C with 8 nM estradiol in Tris buffer containing 0.2 mM PAPS. Each point represents the mean of 2 determinations.


0.10
0.08
0.06
0.04
0.02
0.00
µmol/min
0
200
400
600
800
1000
1200
Protein (µg)

µg/ml of. At a maximum protein concentration of 730 mg/ml more than 60% of the added [^{3}H]-estradiol was converted to $E_2$17S during the 1 h incubation period.

The time course of the sulfoconjugation reaction is shown in Figure 10. The reaction was linear for over 20 min.

The effect of pH on cytosolic E_2-17ß sulfotransferase activity is shown in Figure 11. Using 0.008 mM E_2 and 10 mM Tris buffer, a pH optimum of 7.5 was determined.

Other Effects on E_2-17ß Sulfotransferase Activity

Effect of Cysteine:

A requirement for a free SH group(s) to achieve maximum activity in the case of phenol sulfotransferase (Adams and Poulos, 1967) has been reported. In order to assess the dependence of E_2 17 ß sulfotransferase activity on the presence of sulfhydryl groups, activity was monitored at various concentrations of cysteine. The effect of cysteine on E_2-17ß sulfotransferase activity is shown in Figure 12. $E_2$17ß sulfotransferase activity was overall enhanced by the addition of cysteine. However, a cysteine concentration of 5 mM consistently resulted in a slight decrease in cytosolic E_2-17ß sulfotransferase activity.

Figure 10. E_2-17ß Sulfotransferase Activity as Function of Time.

Cytosol was incubated with estradiol using standard assay conditions as described in Materials and Methods.

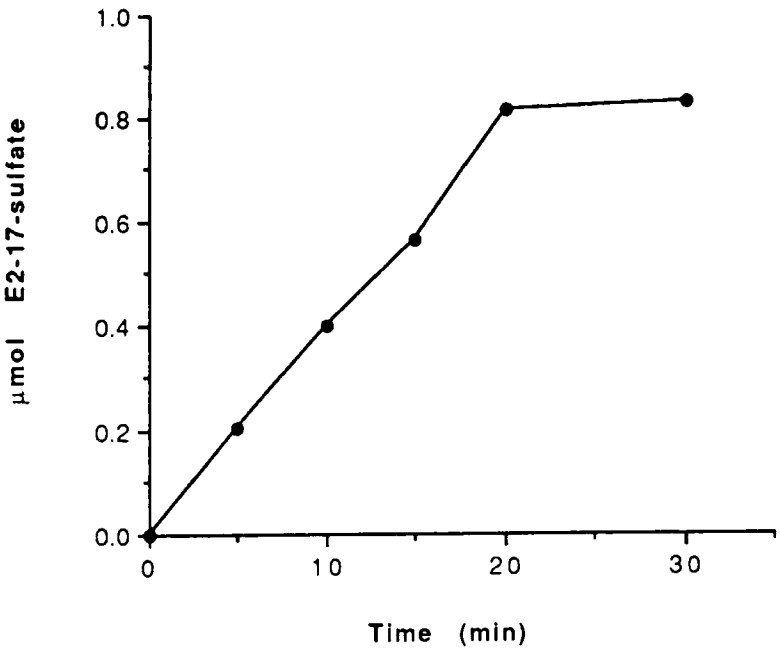

1.0
0.8
0.6
0.4
0.2
0.0
µmol E2-17-sulfate
0
10
20
30
Time (min)

Figure 11. Effect of pH on $E_2$17ß-Sulfotransferase Activity.

Cytosol was incubated with 8 nM estradiol in Tris buffer containing 0.2 mM PAPS. Ionic strength was held constant at 0.12 M by addition of KCl.

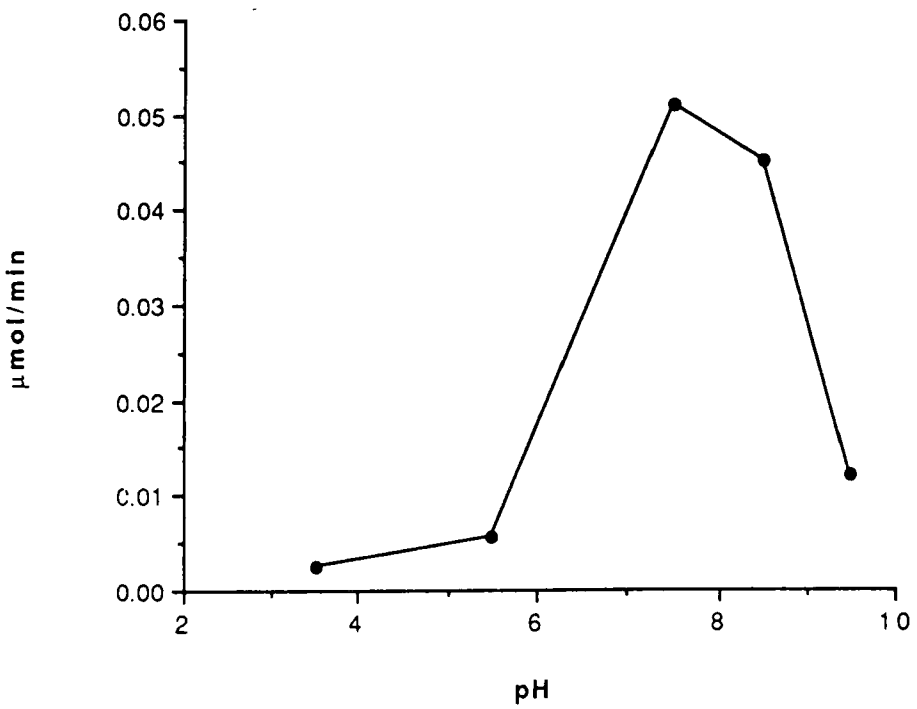
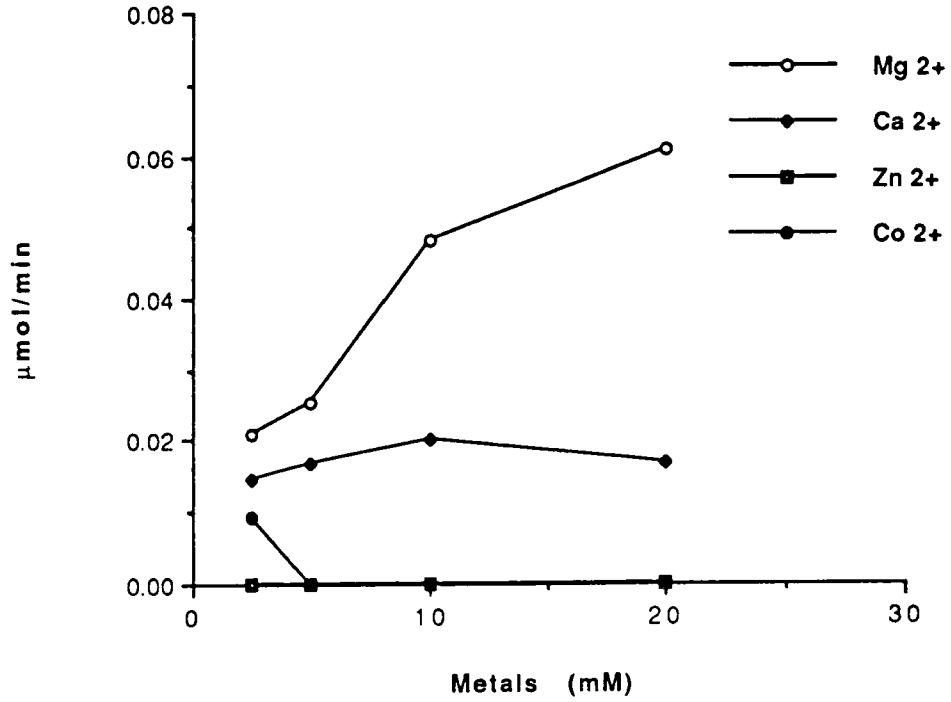

0.06
0.05
0.04
0.03
0.02
0.01
0.00
µmol/min
2
4
6
8
10
pH

Figure 12. Effect of Cysteine on E_2-17ß Sulfotransferase Activity


0.08
0.06
0.04
0.02
0.00
µmol/min
0
10
20
30
Metals (mM)
Mg 2+
Ca 2+
Zn 2+
Co 2+

Effect of Metals

The effect of various metal ions on the activity of E_2-17ß sulfotransferase activity (Figure 13) was very similar to that described for other estrogen sulfotransferases (Adams et al., 1974). At concentration of 0.008 mM E_2 and 0.2 mM PAPS, Mg^{2+} activated 17ß sulfation of E_2. Ca^{2+} enhanced the sulfotransferase activity to a slight extent. Zn^{2+} was strongly inhibitory and Co^{2+} had virtually no effect on E_2-17ß sulfotransferase activity at low concentrations, but was inhibitory at higher concentrations.

Effect of Steroids on Specific Estradiol-17ß Sulfotransferase Activity

These studies were carried out to ascertain whether steroids that inhibited other sulfotransferases affected 17ß sulfation of estradiol. As an index of specificity, test compounds were used at concentrations that gave inhibitor-to-substrate ratios exceeding those resulting in strong inhibition of other sulfotransferases. As shown in the Table 7, at 25 µM estrone, estradiol-17α, and estriol, did not inhibit E_2-17ß sulfotransferase activity, but actually resulted in a significant increase in 17ß sulfation of estradiol ($p < 0.005$). At a concentration of 25 µM, DHEA caused a small but significant ($p < 0.005$) decrease in activity. Testosterone and $E_2$3S present in the

Figure 13. Effect of Metal Ions on $E_2$17ß-Sulfotransferase Activity.

E_2-17ß sulfotransferase activity was measured using the standard sulfotransferase assay at various concentrations (2.0, 5.0, 10.0, 20.0 mM) of metals.

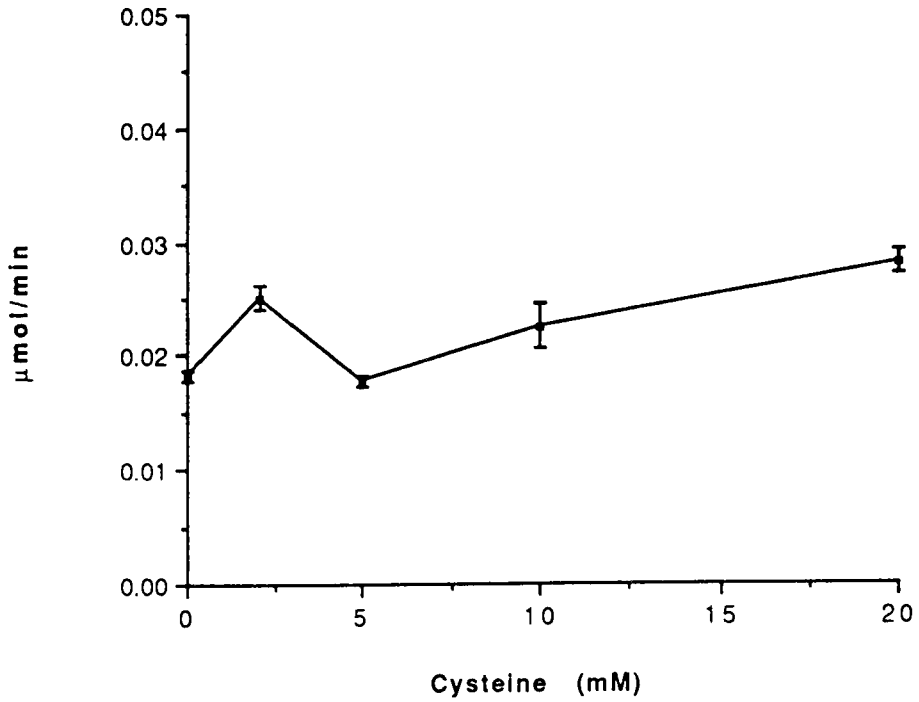

0.05
0.04
0.03
0.02
0.01
0.00
µmol/min
0
5
10
15
20
Cysteine (mM)

Table 7

Effect of Steroids on Estradiol-17ß Sulfotransferase Activity in Squirrel Monkey Liver Cytosol

Steroid	Enzyme activity observed with steroid at indicated concentrations (µM) in reaction mixtures	
	8.0	25.0
Estradiol -17a	12.1±.024	12.7±.003
Estrone	12.0±.016	13.1±.067
Estriol	16.0±.001	15.9±.021
Testosterone	10.0±.048	11.2±.014
DHEA	9.3±.004	9.54±.056
Estradiol-3-sulfate	5.61±.001	5.13±.011

[a] Estradiol-17ß sulfotransferase activity was assayed using the standard enzyme assay with 8 µM estradiol-17ß. An activity of 12.6±.013 µmol/min was determined

[b] These values represent the mean and standard deviation, *n*=3..

assay mixture at equimolar concentrations as E_2 resulted in approximately 30% and 50% inhibition of E_2-17 sulfate production, respectively.

Kinetic Studies

Kinetic studies were conducted with the 140,000 x g supernatant. To determine the affinity of E_2-17ß sulfotransferase for estradiol, estradiol-17ß concentrations were varied while that of PAPS was held constant at 0.2 mM. Figure 14 shows a double reciprocal plot of 1/v versus 1/[E_2]. The apparent K_m determined for E_2-17ß is 5.68 µM. The apparent K_m of E_2-17ß sulfotransferase for PAPS was determined at a fixed E_2 concentration of 0.008 mM over a range of PAPS concentration from 0.005 mM-0.6 mM. A double reciprocal plot of 1/v versus 1/[PAPS] is shown in Figure 15. The calculated K_m for PAPS is 11.6 µM.

Isolation of E_2-17ß Sulfotransferase

The specific activities of E_2-17ß sulfotransferase at various stages of purification are shown in Table 8. A 314-fold purification from the cytosolic preparation with an overall yield of 25% was achieved. Most of the initial sulfotransferase activity present in the crude homogenate was recovered in the cytosolic fraction. Ammonium sulfate

Figure 14. Lineweaver-Burke Plot of E_2-17ß sulfotransferase Activity Versus Concentration of Estradiol-17ß. Apparent K_m =5.68 µM.

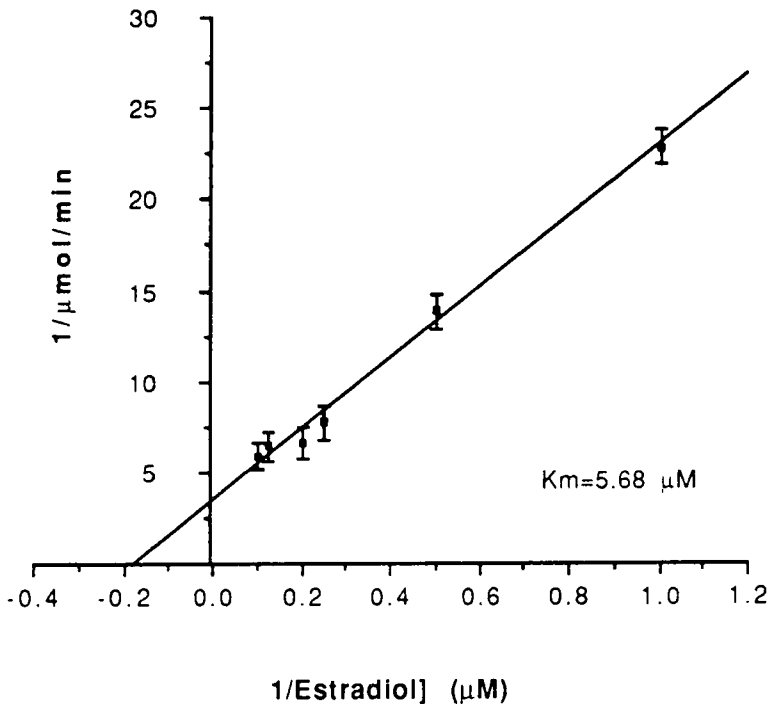

30
25
20
15
10
5
1/µmol/min
Km=5.68 µM
-0.4
-0.2
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1/Estradiol] (µM)

Figure 15. Lineweaver-Burke plot of E_2-17ß Sulfotransferase Activity Versus Concentration of PAPS.

K_m =11.6µM.

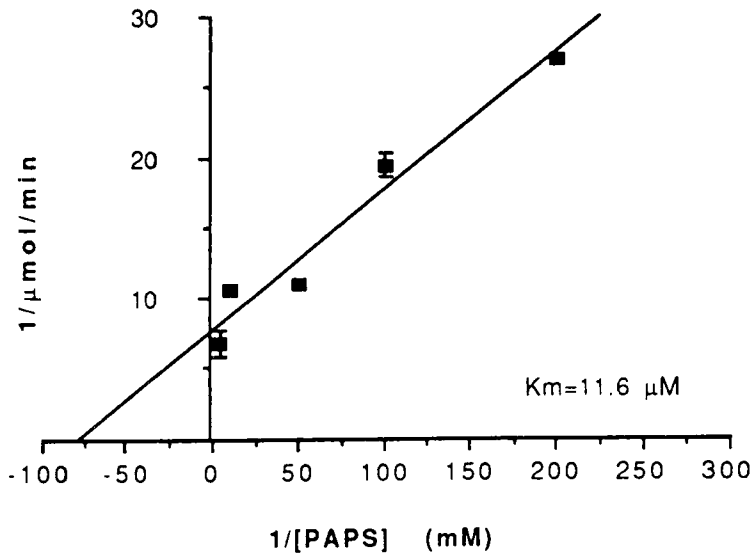

30
20
10
1/μmol/min
Km=11.6 μM
-100
-50
0
50
100
150
200
250
300
1/[PAPS] (mM)

Table 8

Purification of Estradiol-17ß Sulfotransferase in Squirrel Monkey Liver

Preparation	Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Yield (%)	Fold Purified
Homogenate	1096	932	0.85	100	1
Cytosol	450	855	1.9	83	2.2
$(NH4)_2SO_4$ ppt (50%)	105	452	4.3	76	5
DEAE cellulose	35	201	5.8	44	7
PAP-Agarose	0.12	32	267	25	314

[$(NH_4)_2SO_4$] precipitation (50%) was done, and in general, the recovery of the enzyme following this treatment was approximately 76% with a 5-fold purification. Further purification accelerated the loss of E_2-17ß sulfotransferase activity, indicating the unstable nature of the enzyme. Fractionation of the $(NH_4)_2SO_4$ precipitate on a DEAE-Cellulose column is presented in Figure 16. Only one peak of E_2-17ß sulfotransferase activity was detected and the corresponding fractions were pooled and concentrated for further purification by affinity chromatography. As shown in Figure 17, most of the sulfotransferase activity eluted as a single peak after the addition of 0.6 mM PAPS to the elution buffer. However, some sulfotransferase eluted during the washing phase. This low capacity of the affinity column resulted in the substantial loss of enzyme activity during the final purification step.

This study describes an assay of the specific E_2-17ß sulfotransferase delineating optimum assay conditions. Also discussed are several important properties of the enzyme. Subsequent analysis (e.g. kinetic studies, inhibition and substrate specificity) can be performed using purified sulfotransferase to allow a more thorough investigation of the sulfotransferase from squirrel monkey liver.

Figure 16. Chromatography of Estradiol-17ß Sulfotransferase on DEAE-Cellulose. Elution was carried out as described in Materials and Methods. Protein was assayed by continuous monitoring at 280 nm. Fractions were assayed utilizing 8 nM estradiol and 0.2 mM PAPS.

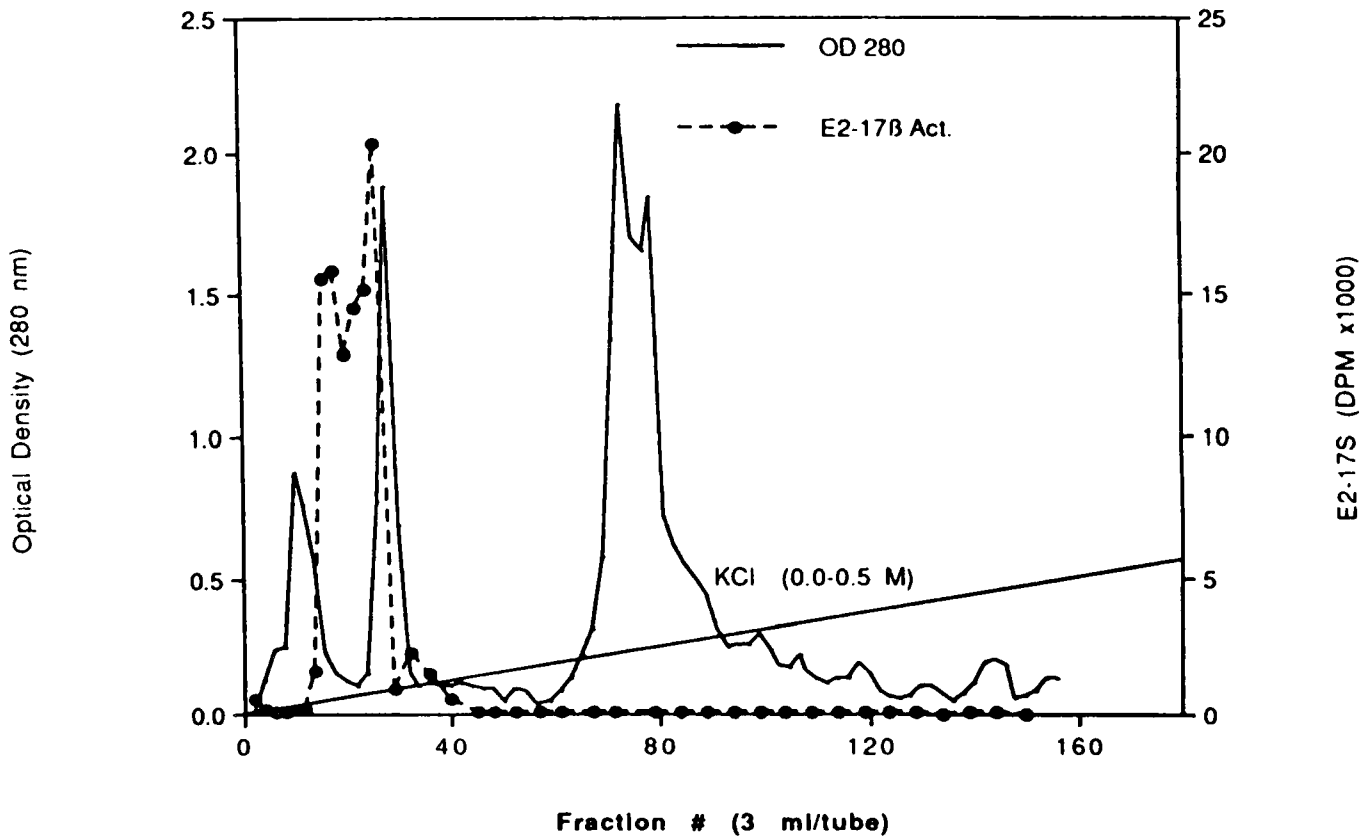
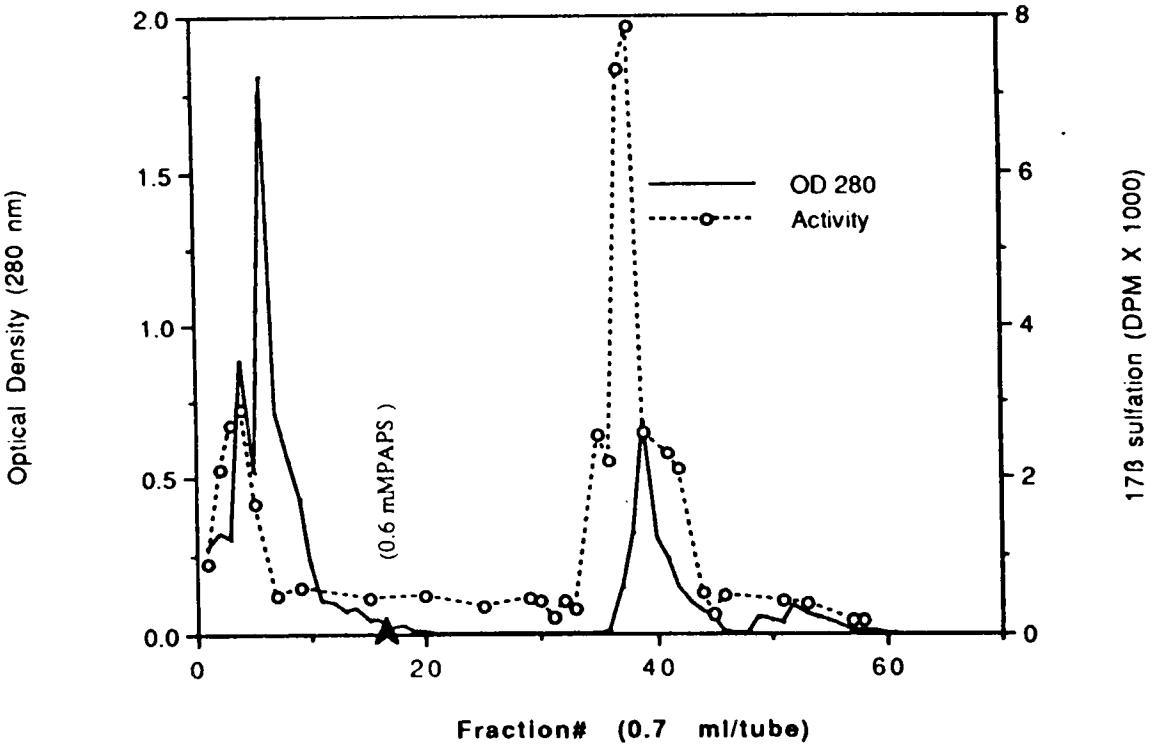

OD 280
E2-17ß Act.
KCl (0.0-0.5 M)
Optical Density (280 nm)
E2-17S (DPM x1000)
Fraction # (3 ml/tube)
2.5
2.0
1.5
1.0
0.5
0.0
25
20
15
10
5
0
0
40
80
120
160

Figure 17. Elution Profile of the Estradiol-17ß Sulfotransferase on Affinity Column, PAP-Agarose. After elution from a DEAE-Cellulose column those fractions with highest E_2-17ß sulfotransferase activity were pooled, concentrated, and chromatographed on a PAP-Agarose column (1 x 15 cm) pre-equilibrated in 50 mM Tris-HCl buffer, 25 mM sucrose, 3 mM ß mercaptoethanol, pH 7.9. Adsorbed protein was eluted by the addition of 0.6 mM PAPS.


Optical Density (280 nm)
2.0
1.5
1.0
0.5
0.0
0
20
40
60
8
6
4
2
0
17β sulfation (DPM X 1000)
OD 280
Activity
(0.6 mMPAPS)
Fraction# (0.7 ml/tube)

CHAPTER V

DISCUSSION

Specific estradiol binding sites were identified in uterine cytosolic and crude nuclear fractions of control and estradiol-primed female squirrel monkeys. The uterine estrogen receptor (ER) level determined in the present study confirms and extends the results of Chrousos et al. (1984) in the only other reported study of squirrel monkey uterine ER determination. While these investigators used ovariectomized animals to eliminate cyclic estradiol and ER changes, the seasonal breeding pattern of this species provided us with squirrel monkeys with low serum E_2 levels equivalent to castrates with respect to circulating estrogen levels. In this study, untreated monkeys had no detectable specific estradiol binding in cytosolic fractions, either occupied or unoccupied by estrogen. Chrousos et al. (1984) using ER assays which measured only unoccupied cytosol ER, found no detectable specific E_2 binders in control animals. These findings demonstrate that when circulating E_2 levels are low, significant cytosol ER concentrations are not maintained in the squirrel monkey cytosol. This contrasts with the rat and

mouse where removal of the ovaries two weeks prior to determination of receptor concentrations results in lower, but still substantial levels of cytosol ER compared to normally cycling animals (Medlock et al., 1981). Results obtained in this study show that unlike in the cytosol, specific estradiol binding sites were detected in the crude nuclear fraction despite low control circulating E_2 levels.

Estrogen treatment induced cytosol ER, demonstrating that the seasonal hiatus in the reproductive cycle does not abolish ER regulation by E_2. However, despite the dramatic increase in ER levels following estrogen priming, these levels are relatively low compared to those in Old World monkeys. Chrousos et al. (1984) found that unoccupied uterine cytosol ER concentrations in the squirrel monkey treated with E_2 were approximately one-third that in similarly dosed cynomolgus monkeys. The dissociation constant for ER binding of E_2 under exchange condition for squirrel monkey cytosol, 2.80±0.24 nM, was essentially identical to the value of 3.0±1 nM reported by Chrousos et al. (1984) under non-exchange conditions. Since uteri were removed 24 h after the last E_2 treatment, the low value for nuclear ER concentration, practically indistinguishable from the control nuclear levels, were expected (Clark et al., 1976).

Data obtained in this study suggest that in the squirrel monkey, elevated plasma estrogen levels are at least in part attributable to decreased receptors in target tissues. In biological systems, generation of estrogenic response is dependent upon the formation of the receptor-hormone complex. The interaction of the steroid and its respective receptor is governed by the classical law of mass action which states that the formation of the receptor-ligand complex is dependent upon the concentration of receptor as well as concentration of the ligand and the affinity of the receptor for its respective ligand. In this study, the squirrel monkey's ER affinity for estradiol was shown to be similar to that of the cynomolgus monkey (Chrousos et al., 1984). Therefore the products of hormone concentration times receptor concentration should be similar and lead to a similar number of ER-complexes, and therefore similar biological effects. To insure a response of equivalent magnitude, low receptor concentration in target tissue would require an increase in plasma steroid concentration. In the squirrel monkey, it is plausible that an increased hormone concentration compensates for a decreased receptor number by increasing receptor occupancy. The increase in plasma hormone levels would insure an equivalent biological response.

The squirrel monkey has been extensively used in experimental research and the results obtained projected to

the human situation. It has been shown by Wolf et al. (1977) that plasma estradiol concentrations are very high compared to that in man and in several Old World primates (Stevens et al., 1970). In addition, other differences have been reported. Chrousos and others (1984) showed that in contrast to Old World species, most of the estrogens in New World monkeys circulates in the free form, unbound to plasma proteins. Pugeat et al. (1984) demonstrated that in the squirrel monkey the concentration and binding affinity of SHBG for estradiol was decreased compared to that in the cynomolgus monkey. Despite these variations in steroid dynamics, the urinary excretion of estrogen conjugates has not been extensively investigated in the squirrel monkey. The present studies were therefore undertaken with the dual objective of increasing existing data on estrogen metabolism in the squirrel monkey and comparing the pattern of estrogen metabolism with those in other primate species.

The most obvious feature of the pattern of estrogen excretion by the squirrel monkey was the small percentage of radiolabeled steroid recovered in the urine. Urinary estrogens collected over the 4 days accounted for slightly less than 5% of the administered radioactive dose. By contrast, urinary excretion represents the principal route of elimination in the human. In this study, estradiol and the 16-keto estrogen comprised the major radiolabeled

metabolites in both the conjugated and unconjugated fractions of urine. Estriol, a principal urinary metabolite in man, was not detected in the urine of the squirrel monkey in this study. Estrone glucosiduronate, the principal urinary estrogen in man, represented a small but significant percentage of the total urinary estrogens.

Most of the estrogens were excreted in the urine without having been conjugated. In this respect, the pattern seen in the squirrel monkey more closely resembles that seen in the baboon (Setchell, 1976). By contrast, only traces of unconjugated estrogens are detected in human urine. The very high level of urinary estradiol found in the squirrel monkey reflects the high plasma free estrogen concentration in this species.

Perhaps one of the most interesting aspects of urinary estrogen excretion in the squirrel monkey was the presence of estradiol-17-sulfate and estradiol-3,17-disulfate in the urine. These two metabolites accounted for most of the excreted estrogens. Detection of estradiol metabolites sulfated at the 17ß position have not been previously reported in the urine of other subhuman primates. A possible significance of this pattern of excretion is to retard the accumulation of toxic concentrations of these compounds. Masking the 17ß hydroxyl group of estradiol greatly reduces its potency. Recently, Watanabe et al.

(1988) have reported the detection of estradiol 17-sulfate in the urine of the human female.

Analysis of fecal excretion yielded striking contrasts as well. As previously alluded to, fecal excretion seems to be the major route of estrogen disposal in the squirrel monkey. This relationship between fecal and urinary estrogen excretion shows strong contrast with the experimental findings of Sandberg and Slaunwhite (1957,1965) who found that approximately 1-18% of intravenously administered ^{14}C-estradiol was excreted in the feces of normal human female subjects.

The pattern of estrogens in feces is unique compared to that found in any other subhuman primate. Of the estrogens found in the feces, only 7% was unconjugated. This contrast markedly with the fecal estrogens of other subhuman primates, 97% of which are unconjugated (Sandberg and Slaunwhite, 1957). It is also of interest that glucosiduronate conjugates were detected in the fecal extracts of the squirrel monkey, whereas in the human female, no glucosiduronates are found in the feces (Eriksson and Gustafsson, 1971). The major fecal metabolite in the squirrel monkey is estradiol-17-sulfate.

The very high levels of fecal estrogens found in this animal reflect a greatly reduced enterohepatic circulation which would tend to increase the time for disposal. In addition, the unusually elevated levels of conjugated

estrogens in the feces imply diminished hydrolytic activity by intestinal microflora. It is also possible that the high proportion of conjugated estrogens is caused by the absence of intestinal bacteria. It is well established that in humans, intestinal microflora are active in deconjugation of steroid sulfates and glucosiduronates (Musey et al., 1972). Furthermore, Adlercreutz et al. (1976) have shown that oral administration of penicillin to pregnant women, which would greatly affect the intestinal microflora, results in an increase in the fecal excretion of both conjugated and unconjugated estriol.

Pack and Brooks (1974) have postulated that estrogen sulfates play a pivotal role in the regulation of the responsiveness of target tissue to circulating hormone. The presence of high levels of $E_2$17S and $E_2$3,17-diS in the plasma, (Musey et al., 1982) urine and feces of the squirrel monkey suggest that sulfoconjugation may serve as a protective mechanism against estrogen toxicity. This conjecture seems particularly valid since there are no known sulfatases capable of converting the conjugate to the free, active estrogen. While sulfatases capable of intracellular hydrolysis of phenolic sulfates to yield bioactive estradiol have been detected, no sulfatases have been detected that hydrolyze the 17ß sulfate moiety. Thus, it is likely that although high plasma estrogen levels are present in the squirrel monkey, steroids circulate

primarily in a form not capable of eliciting classical estrogen responses. Therefore, the preferential 17ß sulfation of estradiol could represent an adaptive mechanism for reducing estrogen exposure.

In the past, investigations have primarily focused on characterization and purification of sulfotransferases specific for the 3ß hydroxyl or phenolic groups of steroids. Adams (1967) purified a sulfotransferase from human adrenal that was specific for estrone, while Singer (1976) isolated a sulfotransferase specific for the 3ß phenolic group of estradiol. Currently, there is no referenced literature on a specific estrogen 17ß sulfurylating enzyme system. This is no doubt attributable to the lack of the detection of estradiol-17ß sulfate products, although Layne et al. (1970) have reported the detection of 17ß sulfated estrogens in urine of laying hen. Recently, Watanabe et al. (1988) reported $E_2$17S in human urine. It should be pointed out, however, that this is the only such report. Because of the potential importance of 17ß sulfation of estradiol in regulation of estrogenicity, it is important that the enzyme system responsible for the 17ß sulfation be further investigated.

Few procedures for the purification of hydroxysteroid sulfotransferase have been reported due to its instability. Adams and MacDonald (1979) however did succeed in isolating a hydroxysteroid sulfotransferase from human adrenals by

affinity chromatography using a DHA-Sepharose gel. Later the purification of three rat liver sulfotransferases of differing specificities were also reported using conventional chromatographic techniques (Lyons and Jacoby, 1980). The low recovery of the partially purified enzyme reported in the present study is similar to that recorded for other liver sulfotransferases. This low yield limited further investigation of enzyme properties. Further work needs to be done on stabilization of the enzyme.

The results obtained in the present study demonstrate that estradiol-17ß sulfurylating activity in squirrel monkey liver cytosol shares several common characteristics with previously described steroid sulfotransferases (Adams and Poulos, 1967; Ryan and Carroll, 1976; Lyons and Jakoby, 1980; Grosso and Way, 1984). The enzyme is localized in the cytosol and the pH optimum (7.5) obtained is within the range reported for other steroid sulfotransferases. Sulfurylation activity is enhanced by cysteine. The decrease in 17ß sulfation in the presence of 5 mM of cysteine (Figure 12) was consistently reproducible. The significance of this effect of cysteine is unknown and requires further investigation. Although an absolute requirement for metal ions was not shown, sulfation was activated by Mg^{2+} and Ca^{2+}, but was strongly inhibited by Zn^{2+} and Co^{2+}.

Sulfation of the 17ß hydroxyl group of estradiol was linear with time as well as with the concentration of protein, but the linearity in relation to the protein concentration could not be extrapolated through the origin. This behavior could be explained by low solubility of estradiol in an aqueous solution in the absence of a critical amount of protein, or the instability of the enzyme at high dilutions as reported for other sulfotransferases (Adams and MacDonald, 1979).

Kinetic studies indicated an apparent K_m of 5.68 µM for estradiol-17ß. This value is within the range the Km of 3-15 µM reported for the bovine adrenal sulfotransferase although, estradiol sulfoconjugation in the squirrel monkey liver did not exhibit the wave-like kinetics characteristic of the bovine adrenal sulfotransferase (Adams et al., 1974). Interestingly, the affinity of the squirrel monkey liver E_2-17ß sulfotransferase for estradiol is markedly greater than that of the rat liver hydroxysteroid sulfotransferase I (Km of 35 µM) reported by Lyons et al. (1980). The rat liver enzyme was also active in sulfation of the 17ß hydroxyl group of estradiol. An apparent K_m of 11.6 µM for PAPS obtained for $E_2$17ß sulfotransferase is similar to the reported affinity for PAPS for other sulfotransferases (Adams et al., 1974).

These investigations indicate very specific steroid substrate requirements of estradiol-17ß sulfotransferase

from squirrel monkey liver homogenate. Unlike the estrogen sulfotransferase of human adrenal which acts upon the phenolic group of estradiol but not estrone, this enzyme system appears to be quite specific for the 17ß hydroxyl group of estradiol. Ring D hydroxyl group in the A configuration as in epimeric estradiol-17α and estrone were not substrates for the enzyme. There were some similarities in specificities with the several hydroxysteroid sulfotransferases from rat liver isolated by Jakoby's group (Lyons and Jakoby, 1980). The latter enzymes sulfated estradiol and not estrone and were reported to catalyze the production of estradiol-17-sulfate. However, DHEA was the preferred substrate, and simple alcohols and other steroids were sulfated to a much greater extent than estradiol. These results suggested that these hydroxysteroid sulfotransferases may represent isozymes as predicted by Ryan and Carroll (1976). The specific estradiol-17ß sulfotransferase of squirrel monkey liver appears to be well suited to convert a large amount of estradiol to estradiol-17-sulfate. In cytosol preparations, estradiol 3ß sulfation was minimal. It is likely that the female squirrel monkey has developed an enzyme system with a greater specificity toward the 17ß hydroxyl group of estradiol to diminish prolonged estrogen exposure.

In summary, the results reported in this study concur with previous findings which suggest that decreased receptor concentrations are responsible for the elevated circulating estrogen levels. Furthermore, various aspects of estrogen metabolism in squirrel monkeys support the contention that elevated estrogen concentrations are maintained as a result of a decreased disposal rate. However, findings in this study also seem to suggest that increased plasma levels are the cause of decreased target tissue sensitivity as well as the altered pathways of estrogen metabolism. Decreased receptor concentration and variations in estrogen metabolism are possible compensatory adaptations to greatly elevated plasma estrogen levels. If alterations of receptor concentration were the cause of elevated estrogen levels, one would expect decreased ER concentrations at the site of regulation not at sensory sites such as the uterus or vagina. MacLusky et al. (1984) have shown that ER concentrations in the pituitary and the brain of the squirrel monkey are comparable to that in the cynomolgus monkey. This observation suggests that the hypothalamic-pituitary system in the squirrel monkey functions in the same fashion as in other primate species. It appears as if the feedback system in the brain and pituitary regulating estradiol-17ß production and secretion is set to a higher estradiol output and is less responsive to negative feedback regulation.

Furthermore, the altered pathway of estrogen metabolism may be a protective mechanism for reducing estrogenicity. Results obtained in this study show an increased fecal excretion of estrogen metabolites with less than 5% excreted via the urine. The high fecal excretion represents a diminished enterohepatic circulation which would diminish the overall exposure of target tissue to the effects of estrogens. Excretion via the kidney would require a longer retention of estrogen metabolites since elimination would be dependent upon glomerular filtration rate. Studies of the effects of manipulations of hormone concentrations on metabolism are needed to determine if alterations in estrogen metabolism is related to elevated plasma E_2 levels.

Results also suggest that in the squirrel monkey, sulfation, particularly 17ß sulfation, is the preferred mechanism of estrogen inactivation. This, as well, is possibly an adaptive mechanism in response to elevated plasma estrogen levels. Sulfation of estradiol at the 17 ß hydroxyl group diminishes estrogen exposure by abolishing the possibility of reactivation of the sulfate. There are no sulfatases that are capable of hydrolysis of the 17ß sulfate moiety. Therefore, although plasma levels of estrogens are high, the predominance of sulfation at C-17ß insures that it circulates in a form not capable of

generating a characteristic estrogenic response. Moreover, other results obtained herein suggest that the squirrel monkey may possess an enzyme system specific for sulfation of steroids at the 17ß hydroxyl group. Further investigation of E_2-17ß sulfotransferase activity using the purified enzyme is necessary to allow a more thorough comparison between this enzyme and other known sulfotransferases.

CHAPTER VI

SUMMARY AND CONCLUSIONS

1. At basal plasma estradiol-17ß concentrations, no specific estradiol binding sites are detected in uterine cytosol of the squirrel monkey.
2. Seasonal hiatus in the reproductive cycle does not abolish the regulation of estrogen receptor levels by estradiol-17ß.
3. Specific estradiol-17ß binding sites are present in nuclear preparations despite non-cycling levels of estradiol-17ß.
4. Estrogen receptor concentrations obtained in the present study support reports that cytosol receptor levels are one-third that in representative Old World primates.
5. The affinity of the estrogen receptor is not significantly different from that reported for Old World monkeys.
6. Patterns of metabolism and excretion of estradiol-17ß in the squirrel monkey are unlike that seen in other non-human primates.

7. Fecal excretion is the priniciple route of elimination of estrogens in the squirrel monkey. This results in a decreased enterohepatic circulation which greatly facilitates rapid excretion.
8. The result of *in vivo* metabolic studies indicate that sulfation is the primary conjugation mechanism, with estradiol-17ß-sulfate as the major metabolite (accounting for 55% of the injected radioactivity).
9. Glucosiduronate conjugates of estradiol which represent the principle urinary metabolites in man and other primates, accounted for only 26% of the radiolabeled estrogen excreted by the squirrel monkey.
10. Two metabolites, estradiol-17ß-sulfate and estradiol-3ß,17ß-disulfate, not previously identified in the urine and feces of other sub-human primates, represented a large percentage of the excreted tritium-labeled metabolites in squirrel monkey urine and feces.
11. The enzyme responsible for the specific 17ß sulfation of estradiol has been characterized in the squirrel monkey liver cytosol.
12. A 314-fold purification of estradiol-17ß sulfotransferase from squirrel monkey liver was achieved with a final yield of 25%.

13. The enzyme system appears to be distinct from the estrogen sulfotransferase previously described by Adams et al. (1974) that specifically recognized the 3ß-hydroxyl group of estrone and estradiol.
14. Maximum sulfation of the 17ß hydroxyl group of estradiol was obtained at pH 7.5.
15. Estradiol-17ß-sulfate formation was enhanced by the presence of cysteine, Mg^{2+} and Ca2+, but was inhibited by Zn^{2+} and Co^{2+}.
16. Estradiol-17ß-sulfate formation was partially inhibited by 17ß hydroxysteroids but not by estrone and 17α-estradiol.
17. The apparent K_ms of the enzyme for estradiol and 3'-phosphoadenosine-5'-phosphosulfate were 5.68 and 11.6 μM, respectively.

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